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PRACTICAL
PHYSIOLOGICAL CHEMISTRY

HAWK

Absorption Spectra.

PLATE I.

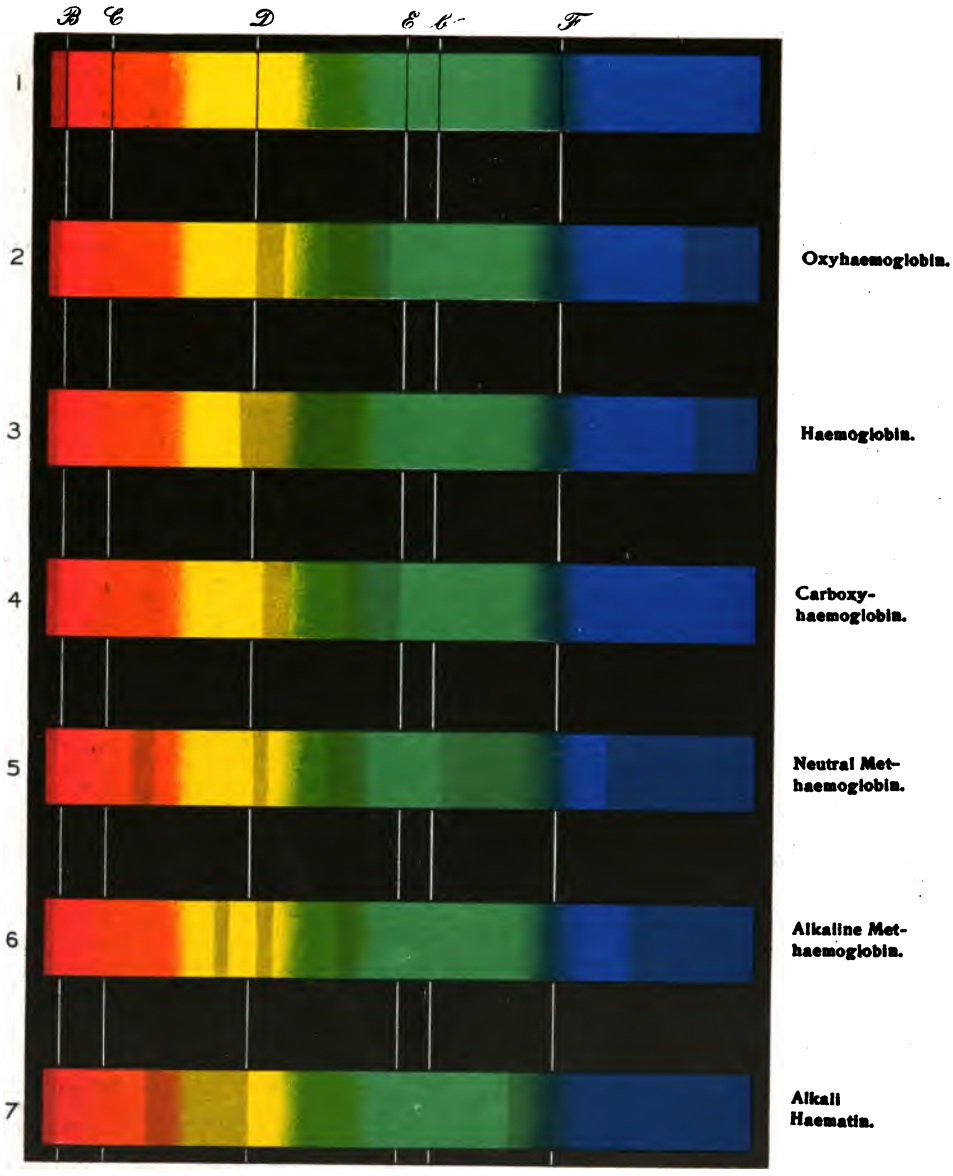

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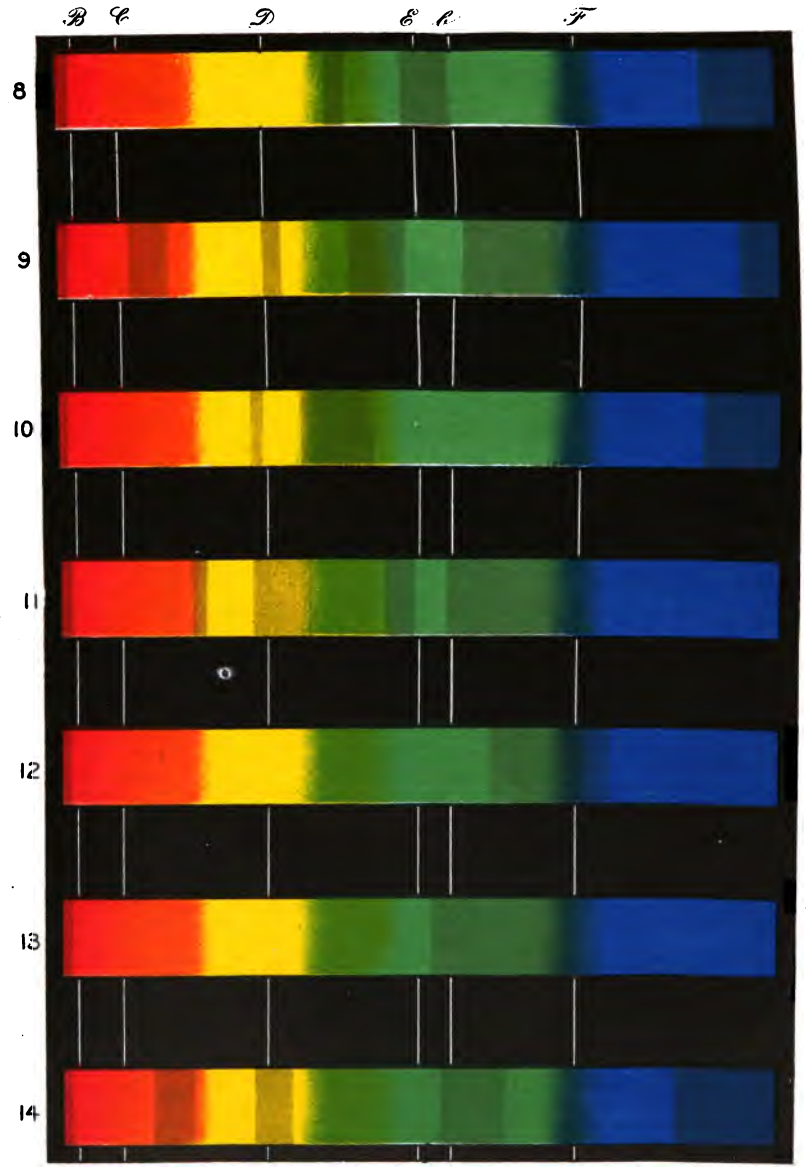
PLATE II.



PRACTICAL

PHYSIOLOGICAL CHEMISTRY

A BOOK DESIGNED FOR USE IN COURSES IN PRACTICAL PHYSIOLOGICAL CHEMISTRY IN SCHOOLS OF MEDICINE AND OF SCIENCE

BY

PHILIP B. HAWK, M. S., Ph. D.

PROFESSOR OF PHYSIOLOGICAL CHEMISTRY IN THE UNIVERSITY OF ILLINOIS

THIRD EDITION, REVISED AND ENLARGED

WITH TWO FULL-PAGE PLATES OF ABSORPTION SPECTRA IN COLORS, FOUR ADDITIONAL FULL-PAGE COLOR PLATES AND ONE HUNDRED AND TWENTY-SEVEN FIGURES OF WHICH TWELVE ARE IN COLORS

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AFFECTIONATELY DEDICATED

TO

MY MOTHER

PREFACE TO THIRD EDITION

The increasing approval with which this volume is being received has rendered necessary the preparation of a new edition, although the period elapsing since the last edition appeared is little more than one year. The present edition has been brought up to date by the insertion of various additions and corrections as well as by the inclusion of a number of qualitative tests and quantitative methods. Because of the very short intervening period since the last edition of the volume, the new material inserted is rather small in quantity when compared with that incorporated at the previous revision.

The author wishes to thank Dr. W. H. Welker and Dr. Croll for permission to insert unpublished material.

URBANA, *September*, 1910.

PREFACE TO SECOND EDITION

The kind reception accorded this volume by the instructors in physiological chemistry in the United States and Great Britain has made the preparation of a new edition imperative, notwithstanding the fact that less than two years have elapsed since the former edition appeared. The advance and development made in the field of physiological chemistry during this period have been both rapid and important; conditions which would of themselves have necessitated the revision of the volume at an early date.

The book has been thoroughly revised in all departments and in part rewritten, the system of spelling officially adopted by the American Chemical Society having been followed throughout the volume. Besides introducing many new qualitative tests and quantitative methods, the author has added a chapter on "Enzymes and Their Action" and has rewritten the two chapters on Proteins. The term "protein" has been substituted for "proteid" and the classification of proteins as recently adopted by the American Physiological Society and the American Society of Biological Chemists has been introduced and is followed throughout the text; the classification adopted by the Chemical and Physiological Societies of England is also included.

The original plan of the book has been adhered to with the exception that the chapter on "Enzymes and Their Action" has been made Chapter I and the practical work upon the proteins is preceded by a chapter giving a brief discussion of protein substances from the standpoint of their decomposition and synthesis. We believe that the student will be able to pursue his practical work more intelligently and will derive greater benefit therefrom if the plan of instruction as suggested in Chapters IV and V be followed in the presentation of the subject of "Proteins."

The author wishes to express his thanks to all those who so kindly offered suggestions for the betterment of the book. He is particularly desirous of expressing his gratitude to Professor Lafayette B. Mendel and Dr. Thomas B. Osborne for the many helpful suggestions they have so kindly given him. His thanks are also due Professor C. A. Herter, Dr. H. D. Dakin, Dr. S. R. Benedict, and Mr. S. C. Clark

for permission to insert unpublished material, to Mr. Paul E. Howe for valuable assistance rendered in the reading of proof and in the verification of tests and methods, and to Dr. M. E. Rehfuss for assistance in proof reading.

The author takes this opportunity of making an acknowledgment which was inadvertently omitted from the first edition. He wishes to express his obligation to the laboratories of physiological chemistry at Yale University and at Columbia University (College of Physicians and Surgeons) in the latter of which he was Assistant to Professor W. J. Gies for two years. The courses given in these laboratories formed the basis of many of the experiments included in this volume, and it is with feelings of deepest gratitude that he records this acknowledgement of the assistance thus rendered by those in charge of these courses.

PHILIP B. HAWK.

URBANA, ILLINOIS,
February, 1909.

PREFACE TO FIRST EDITION

The plan followed in the presentation of the subject of this volume is rather different, so far as the author is aware, from that set forth in any similar volume. This plan, however, he feels to be a logical one and has followed it with satisfactory results during a period of three years in his own classes at the University of Pennsylvania. The main point in which the plan of the author differs from those previously proposed is in the treatment of the food stuffs and their digestion.

In Chapter IV the "Decomposition Products of Proteids" has been treated although it is impracticable to include the study of this topic in the ordinary course in practical physiological chemistry. For the specimens of the decomposition products, the crystalline forms of which are reproduced by original drawings or by micro-photographs, the author is indebted to Dr. Thomas B. Osborne of New Haven, Conn.

Because of the increasing importance attached to the examination of feces for purposes of diagnosis, the author has devoted a chapter to this subject. He feels that a careful study of this topic deserves to be included in the courses in practical physiological chemistry, of medical schools in particular. The subject of *solid tissues* (Chapters XIII, XIV and XV) has also been somewhat more fully treated than has generally been customary in books of this character.

The author is deeply indebted to Professor Lafayette B. Mendel, of Yale University, for his careful criticism of the manuscript and to Professor John Marshall, of the University of Pennsylvania, for his painstaking revision of the proof. He also wishes to express his gratitude to Dr. David L. Edsall for his criticism of the clinical portion of the volume; to Dr. Otto Folin for suggestions regarding several of his quantitative methods, and to Mr. John T. Thomson for assistance in proof reading.

For the micro-photographs of oxyhæmoglobin and hæmin reproduced in Chapter XI the author is indebted to Professor E. T. Reichert, of the University of Pennsylvania, who, in collaboration with Professor A. P. Brown, of the University of Pennsylvania, is making a very ex-

tended investigation into the crystalline forms of biochemic substances. The micro-photograph of allantoin was kindly furnished by Professor Mendel. The author is also indebted for suggestions and assistance received from the lectures and published writings of numerous authors and investigators.

The original drawings of the volume were made by Mr. Louis Schmidt whose eminently satisfactory efforts are highly appreciated by the author.

PHILIP B. HAWK.

PHILADELPHIA.

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PHYSIOLOGICAL CHEMISTRY.

CHAPTER I.

ENZYMES AND THEIR ACTION.

ACCORDING to the old classification ferments were divided into two classes, the *organized ferments* and the *unorganized ferments*. As organized ferments or true ferments there were grouped such substances as *yeast* and certain *bacteria* which were supposed to act by virtue of vital processes, whereas the unorganized ferments included *salivary amylase* (ptyalin), *gastric protease* (pepsin), *pancreatic protease* (trypsin), etc., which were described as "non-living unorganized substances of a chemical nature." Kühne designated this latter class of substances as *enzymes* (ἐν ζύμη—in yeast). This division into organized ferments (true ferments) and unorganized ferments (enzymes) was generally accepted and was practically unquestioned until Buchner overthrew it in the year 1897 by his epoch-making investigations on zymase. Previous to this time many writers had expressed the opinion that the action of the ferment organisms was similar to that of the unorganized ferments or enzymes and that therefore the activity of the former was possibly due to the production of a substance in the cell, which was in nature similar to an enzyme. Investigation after investigation, however, failed to isolate any such principle from an active cell and the exponents of the "vital" theory became strengthened in their belief that certain fermentative processes brought about by living cells could not occur apart from the biological activity of such cells. However, as early as 1858, Traube had enunciated, in substance, the principles which were destined to be fundamental in our modern theory of fermentation. He expressed the belief that the yeast cell produced a product in its metabolic activities which had the property of reacting with sugar with the production of carbon dioxide and alcohol, and further that this reaction between the product of the metabolism of the yeast cell and the sugar occurred *without aid from the original cell*. It was not until 1897, however, that this theory was placed upon a firm

experimental basis. This was brought about through the efforts of Buchner who succeeded in isolating from the living yeast cells a substance (zymase) which, when freed from the last trace of organized cellular material, was able to bring about the identical fermentative processes, which, up to this time, had been deemed possible only in the presence of the active, living yeast cell.

Buchner's manipulation of the yeast cells consisted in first grinding them with sand and infusorial earth, after which the finely divided material was subjected to great pressure (300 atmospheres) and yielded a liquid which possessed the fermentative activity of the unchanged yeast cell.[1] This liquid contained *zymase*, the principal enzyme of the yeast cell. Later the lactic-acid- and acetic-acid-producing bacteria were subjected by Buchner to treatment similar to that accorded the yeast cells, and the active intracellular enzymes were obtained. Many other instances are on record in which a soluble, active agent has been isolated from ferment cells, with the result that it is pretty well established that all the so-called organized ferments elaborate substances of this character. Therefore, basing our definition on the work of Buchner and others we may define an enzyme as *an unorganized, soluble ferment, which is elaborated by an animal or vegetable cell and whose activity is entirely independent of any of the life processes of such a cell.* According to this definition the enzyme *zymase* elaborated by the yeast cell is entirely comparable to the enzyme *pepsin* elaborated by the cells of the stomach mucosa. One is derived from a vegetable cell, the other from an animal cell, yet the activity of neither is dependent upon the integrity of the cell.

Enzymes act by *catalysis* and hence may be termed catalyzers or catalysts. A simple rough definition of a catalyzer is "a substance which alters the velocity of a chemical reaction without undergoing any apparent physical or chemical change itself and without becoming a part of the product formed." It is a well-known fact that the velocity of the greater number of chemical reactions may be changed through the presence of some catalyzer. For example, take the case of hydrogen peroxide. It spontaneously decomposes slowly into water and oxygen. In the presence of colloidal platinum,[2] however, the decomposition is much accelerated and ceases only when the destruction of the hydrogen peroxide is complete. Without multiplying instances, suffice it to say that there is an analogy between inorganic catalyzers

[1] In later investigations the process was improved by freezing the ground cells with liquid air and finely pulverizing them before applying the pressure.

[2] Produced by the passage of electric sparks between two platinum terminals immersed in distilled water, thus liberating ultra-microscopic particles.

and enzymes, the main point of difference between the enzymes and most of the inorganic catalyzers being that the enzymes are colloids.[1]

Inasmuch as each of the enzymes has an action which is more or less specific in character, and since it is a fairly simple matter, ordinarily, to determine the character of that action, the classification of the enzymes is not attended with very great difficulties. They are ordinarily classified according to the nature of the substrate[2] or according to the type of reaction they bring about. Thus we have various classes of enzymes, such as *amylolytic*,[3] *proteolytic*, *lipolytic*, *glycolytic*, *uricolytic*, *autolytic*, *oxidizing*, *reducing*, *inverting*, *protein-coagulating*, *deamidizing*, etc. In every instance the class name indicates the individual type of enzymatic activity which the enzymes included in that class are capable of accomplishing. For example, amylolytic enzymes facilitate the hydrolysis of starch (amylum) and related substances, lipolytic enzymes facilitate the hydrolysis of fats (λιπος), whereas through the agency of uricolytic enzymes uric acid is broken down. There is a tendency, at the present time, to harmonize the nomenclature of the enzymes by the use of the termination, *-ase*. According to this system of nomenclature, all starch-transforming enzymes, or so-called amylolytic enzymes, are called *amylases*, all fat-splitting enzymes are called *lipases*, etc. Thus *ptyalin* the amylolytic enzyme of the saliva, would be termed *salivary* amylase in order to distinguish it from *pancreatic* amylase (amylopsin) and *vegetable* amylases (diastase, etc.). According to the same system, the fat-splitting enzyme of the gastric juice would be termed *gastric* lipase to differentiate it from *pancreatic* lipase (steapsin), the fat-splitting enzyme of the pancreatic juice.

Our knowledge regarding the distribution of enzymes has been wonderfully broadened in recent years. Up to within a few years, the real scientific information as to the enzymes of the animal organism, for example, was limited, in the main, to a rather crude understanding of the enzymes intimately connected with the main digestive functions of the organism. We now have occasion to believe that enzymes are doubtless present in every animal cell and are actively associated with all vital phenomena. As a preëminent example of such cellular activity may be cited the liver cell with its reputed complement of 15–20 or more enzymes.

[1] Bredig has been able to obtain certain inorganic catalyzers in *colloidal solution*. These he calls "*inorganic enzymes*."

[2] Substance acted upon.

[3] Armstrong suggests the use of the termination "clastic" instead of "lytic." He calls attention to the fact that *amylolytic*, in analogy with *electrolytic*, means "decomposition by means of starch" and is therefore a misnomer. He suggests the use of *amyloclastic*, *proteoclastic*, etc.

In text-book discussions of the enzymes it is customary to say that very little is known regarding the chemical characteristics of these substances since no member of the enzyme group has, up to the present time, been prepared in an *absolutely pure condition.* Apparently, however, from the nature of the facts in the case, it would be much more accurate to say that we absolutely *do not know* whether a specific enzyme *has, or has not,* been prepared in a pure state. (Some authors, like Arthus, have assumed that enzymes are not chemical individuals, but *properties conferred upon bodies.*) The enzymes are very difficult to prepare in anything like a condition approximating purity, since they are very prone to change their nature during the process by which the investigator is attempting to isolate them. For this reason we have absolutely no proof that the final product obtained is, or is not, in the same state of purity it possessed in the original cell. Some of the enzymes are more or less closely associated with the proteins from the fact that they are both formed in every cell as the result of the cellular activity, both may be removed from solution by "salting-out," both are for the most part non-diffusible and are probably very similar as regards elementary composition. Hence in the preparation of some enzymes it is extremely difficult to make an absolute separation from the protein.[1] Under certain conditions enzymes are readily adsorbed by shredded protein material, such as fibrin, and may successfully resist the most prolonged attempts at washing them free. We may summarize some of the properties of the great body of enzymes as follows: Enzymes are soluble in *dilute* glycerol, sodium chloride solution, *dilute* alcohol and water, and precipitable by ammonium sulphate and *strong* alcohol. Their presence may be proven from the nature of the end-products of their action and not through the agency of any chemical test. They are *colloidal* and *non-diffusible,* and occur closely associated with protein material with which they possess many properties in common. Each enzyme shows the greatest activity at a certain temperature called the *optimum* temperature; there is also a *minimum* and a *maximum* temperature for each specific enzyme. Their action is inhibited by sufficiently lowering the temperature, and the enzyme, if in solution, is entirely destroyed by subjecting it to a temperature of 100° C. The best known enzymes, whether derived from warm-blooded or cold-blooded animals, are most active between 35°–45° C. The nature of the surrounding media alters the velocity of the enzymatic action, some enzymes being more active in acid solution whereas others require an alkaline fluid.

[1] Others seem to be like the substrate on which they act, *e. g.*, carbohydrate.

Many of the more important enzymes do not occur preformed within the cell, but are present in the form of a *zymogen* or mother-substance. In order to yield the active enzyme this zymogen must be transformed in a certain specific manner and by a certain specific substance. This transformation of the inactive zymogen into the active enzyme is termed *activation*. For instance, the zymogen of the enzyme pepsin of the gastric juice, termed *pepsinogen*, is activated by the *hydrochloric acid* secreted by the gastric cells (see p. 116), whereas the activation of the *trypsinogen* of the pancreatic juice is brought about by a substance termed *enterokinase*[1] (see p. 138). These are examples of many well-known activation processes going on continually within the animal organism. The agency which is instrumental in activating a zymogen is generally termed a *zymo-exciter* or a *kinase*. In the cases cited hydrochloric acid would be termed a zymo-exciter and enterokinase would be termed a kinase.

After filtering yeast juice, prepared by the Buchner process (see p. 2), through a Martin gelatin filter, Harden and Young showed that the colloids left behind and the filtrate were both inactive fermentatively. Upon treating the colloid material (enzyme) with some of the filtrate, however, the mixture was shown to be able to bring about pronounced fermentation. It is believed that a *co-enzyme* present in the filtrate was the efficient agent in the transformation of the inactive enzyme. It is necessary to make frequent renewals of the co-enzyme in order to maintain continuous fermentation. It was further shown that this co-enzyme, in addition to being diffusible, was not destroyed by boiling and that it disappeared from yeast juice when this latter was fermented or allowed to undergo autolysis. The exact nature of this co-enzyme of zymase is unknown. The co-enzyme action, in this case, is probably dependent upon the presence of two individual agencies, one of which is *phosphates*.

It has been shown by Loevenhart that the property of acting as a pancreatic lipase co-enzyme is vested in *bile salts*, and Magnus has further shown that the synthetic salts are as efficient in this regard as the natural ones. A few other instances of co-enzyme demonstrations have been reported.

The so-called "specificity" of enzyme action is an interesting and important fact. That enzymes are very specific as to the character of the *substrate*, or substance acted upon, is well known. Emil Fischer investigated this problem of specificity extensively in connection with

[1] According to Delezenne, trypsinogen may be rapidly activated by soluble calcium salts.

the fermentation of sugars and reached the conclusion that enzymes, with the possible exception of certain oxidases, can act only upon such substances as have a specific stereo-isomeric relationship to themselves. He considers that the enzyme and its substrate must have an interrelation, *such as the key has to the lock*, or the reaction does not occur. Fischer was able to predict, in certain definite cases, from a knowledge of the constitution and stereo-chemical relationships of a substance, whether or not it would be acted upon by a certain enzyme. An application of this specificity of enzyme action may be seen in the well-known facts that certain enzymes act on carbohydrates, others on fats, and others on protein; and, moreover, that the group of those which transform carbohydrates, for example, is further subdivided into specific enzymes each of which has the power of acting alone upon some one sugar.

It has been conclusively shown, in the case of certain enzymes,[1] at least, that their action is a *reversible* one and is, in all its main features, directly analogous to the reversible reactions produced by chemical means. For instance, in the saponification of ethyl-butyrate by means of pancreatic lipase, it has been shown that upon the formation of the end-products of the reaction, *i. e.*, butyric acid and ethyl alcohol, there is reversion[2] and the reaction is stationary. This does not mean there are no chemical changes going on, but simply indicates that chemical *equilibrium* has been established, and that the change in one direction is counterbalanced by the change in the opposite direction. Pancreatic lipase was one of the first enzymes to have the reversibility of its reaction clearly demonstrated.[3] A knowledge of the fact that lipase possesses this reversibility of action is of extreme physiological importance and aids us materially in the explanation of the processes involved in the digestion, absorption, and deposition of fats in the animal organism (see p. 130).

In respect to many enzymes it has been found that the law governing the action of inorganic catalyzers is directly applicable, *i. e.*, that the *intensity is almost directly proportional to the concentration of the enzyme.* In the case of enzymes, however, there is a difference in that a maximum intensity is soon reached and that subsequent concentration of the enzyme is productive of no further increase in intensity.

[1] This is probably a general condition.

[2] The re-synthesis of ethyl-butyrate from its hydrolysis products. This may be indicated thus:

$$C_3H_7COO.C_2H_5 + H_2O \rightleftarrows C_3H_7COOH + C_2H_5OH.$$

Ethyl butyrate. *Butyric acid. Ethyl alcohol.*

[3] The principle was *first* demonstrated in connection with the enzyme maltase (see p. 54).

The enzymes which have been shown to obey this *linear law* are lipase, invertase, rennin, and trypsin. In *certain instances*, where this law of direct proportionality between the intensity of action and the concentration of enzyme does not hold, it has been found that the *law of Schütz*, first experimentally demonstrated by E. Schütz, was applicable. This is to the effect that the intensity is directly proportional to the square root of the concentration, or conversely, that *the relative concentrations of enzymes are directly proportional to the squares of the intensities.*[1]

It has been shown that there are certain substances which possess the property of directly inhibiting or preventing the action of a catalyzer. These are called *anti-catalyzers* or paralyzers and have been compared to the *anti-toxins*. Related to this class of anti-catalytic agents stand the *anti-enzymes*. The first anti-enzyme to be reported was the *anti-rennin* of Morgenroth. This was produced by injecting into an animal increasing doses of rennet solution, whereupon an "anti" substance was subsequently found both in the serum and in the milk, which prevented the enzyme rennin from exerting its normal activity in the presence of caseinogen. In other words, *anti-rennin* had been formed in the serum of the animal,[2] through the repeated injections of rennet solution. Since the discovery of this anti-enzyme, anti-bodies have been demonstrated for pepsin, trypsin, lipase, urease, amylase, laccase, tyrosinase, emulsin, papain, and thrombin. According to Weinland, the reason why the stomach does not digest itself is, that during life there is present in the mucous membrane of the stomach an *anti-enzyme* (*anti-pepsin*) which has the property of inhibiting the action of pepsin. A similar substance (anti-trypsin) is present in the intestinal mucosa as well as in the tissues of various intestinal worms. Some investigators are not inclined to accept the enzyme nature of these inhibitory agents as proven.

EXPERIMENTS ON ENZYMES AND ANTI-ENZYMES.

A. Experiments on Enzymes.[3]

I. AMYLASES.

1. **Demonstration of Salivary Amylase.**[4]—To 25 c.c. of a one per cent starch paste in a small beaker, add 5 drops of saliva and stir

[1] This law of Schütz is not generally applicable.

[2] Serum is normally *anti-tryptic*.

[3] If it is deemed advisable by the instructor to give all the practical work upon enzymes at this point in the course, additional experiments will be found in Chapters III, VI and VIII.

[4] For a discussion of this enzyme see p. 53.

thoroughly. At intervals of a minute remove a drop of the solution to one of the depressions of a test-tablet and test by the iodine test.[1] If the blue color with iodine still forms after five minutes, add another five drops of saliva. The opalescence of the starch solution should soon disappear, indicating the formation of *soluble starch* (*amidulin*) which gives a blue color with iodine. This body should soon be transformed into *erythrodextrin* which gives a red color with iodine, and this, in turn, should pass into *achroodextrin* which gives *no color* with iodine. This point is called the *achromic point.* When this point is reached test by Fehling's test[2] to show the production of a reducing substance (maltose). A positive Fehling's test may be obtained while the solution still reacts red with iodine inasmuch as some sugar is formed from the soluble starch coincidently with the formation of the erythrodextrin. For further discussion of the transformation of starch see p. 53.

2. **Demonstration of Pancreatic Amylase.**[3]—Proceed exactly as indicated above in the Demonstration of Salivary Amylase except that the saliva is replaced by 5 c.c. of pancreatic extract prepared as described on p. 141. Pancreatic amylase transforms the starch in a manner entirely analogous to the transformation resulting from the action of salivary amylase.

3. **Preparation of Vegetable Amylase.**—Extract finely ground malt with water, filter and subject the filtrate to alcoholic fermentation by means of yeast. When fermentation is complete filter off the yeast and precipitate the amylase from the filtrate by the addition of alcohol. The precipitate may be filtered off and obtained in the form of a fine white powder.

4. **Demonstration of Vegetable Amylase.**—This enzyme may be demonstrated according to the directions given under Demonstration of Salivary Amylase, p. 7, with the exception that the saliva used in that experiment is replaced by an aqueous solution of the vegetable amylase powder prepared as described above.[4]

II. PROTEASES.

1. **Preparation of Gastric Protease.**[5]—Treat the finely comminuted mucosa of a pig's stomach with 0.4 per cent hydrochloric

[1] See p. 45.
[2] See p. 27.
[3] For a discussion of this enzyme see p. 139.
[4] If desired the *first* aqueous extract of the original malt may be used in this demonstration. Commercial *taka-diastase* may also be employed.
[5] Also called *pepsin*, *pepsase*, *gastric proteinase*, and *acid protease*. For a discussion of this enzyme see p. 117.

acid and extract at 38° C. for 24–48 hours. The filtrate from this mixture constitutes a very satisfactory acid extract of gastric protease (see p. 119).

2. **Demonstration of Gastric Protease.**—Introduce some protein material (fibrin, coagulated egg-white, or washed lean beef) into the acid extract of gastric protease prepared as above described,[1] add an equal volume of 0.4 per cent hydrochloric acid and place the mixture at 38° C. for 2–3 days. Identify the products of digestion according to directions given on p. 119.

3. **Preparation of Pancreatic Protease.**[2]—A satisfactory extract of this enzyme may be made from the pancreas of a pig or sheep according to the directions given on p. 141.

4. **Demonstration of Pancreatic Protease.**—Into an alkaline extract of pancreatic protease,[3] prepared as directed on p. 141, introduce some fibrin, coagulated egg-white or lean beef and place the mixture at 38° C. for 2–5 days.[4] At the end of that period separate and identify the end-products of the action of pancreatic protease according to the directions given on p. 141.

5. **Demonstration of a Vegetable Protease.**—A commercial preparation of *papain* (*papayotin*, *carase* or *papase*), the protease of the fruit of the pawpaw (*carica papaya*), may be used in this connection. Follow the same procedure as that described under Gastric Protease (see above).

III. LIPASES.

1. **Preparation of Pancreatic Lipase.**[5]—An extract of this enzyme may be prepared from the pancreas of the pig or sheep according to the directions given on p. 141.[6]

2. **Demonstration of Pancreatic Lipase.**—Into each of two test-tubes introduce 10 c.c. of milk and a small amount of litmus powder. To the contents of one tube add 3 c.c. of a *neutral* extract of *pancreatic lipase* and to the contents of the other tube add 3 c.c. of a *boiled* neutral extract of *pancreatic lipase*. Keep the tubes at 38° C. and watch for color changes. The blue color of the litmus

[1] If so desired, a solution of commercial pepsin powder in 0.2 per cent hydrochloric acid may be substituted.

[2] Also called *trypsin*, *trypsase*, *pancreatic proteinase* and *alkali proteinase*. For a discussion of this enzyme see p. 128.

[3] A 0.25 per cent sodium carbonate solution of commercial *trypsin* may be substituted.

[4] A few c.c. of toluene or an alcoholic solution of thymol should be added to prevent putrefaction.

[5] Also called steapsin. For a discussion of this enzyme see p. 139. A very active lipolytic extract may also be prepared from the liver.

[6] If preferred, a *glycerol extract* may be prepared according to the directions given by Kanitz; *Zeitschrift für physiologische Chemie*, 1906, XLVI, p. 482.

powder will gradually give place to a red. This change in the color of the litmus from blue to red has been brought about by the fatty acid which has been produced through the lipolytic action exercised by the lipase upon the milk fats.

3. **Preparation of Vegetable Lipase.**—This enzyme may be readily prepared from castor beans, several months old, by the following procedure:[1] Grind the shelled beans very fine[2] and extract for twenty-four-hour periods with alcohol-ether and ether, in turn. Reduce the semi-fat-free material to the finest possible consistency by means of mortar and pestle and pass this material through a sieve of very fine mesh. Place this material in a Soxhlet extractor and extract for one week. This fat-free powder may then be used to demonstrate the action of vegetable lipase. Powder prepared as described may be used in quantitative tests. For ordinary qualitative tests it is not necessary to remove the last traces of fat and therefore the extraction period in the Soxhlet apparatus may be much shortened.

4. **Demonstration of Vegetable Lipase.**—The lipolytic action of the lipase prepared from the castor bean, as just described, may be demonstrated in a manner entirely analogous to that used in the Demonstration of Pancreatic Lipase, see p. 9. Proceed as indicated in that experiment and substitute the vegetable lipase powder for the neutral extract of pancreatic lipase. The type of action is entirely analogous in the two instances.

An experiment similar to Experiment 2, p. 145, may also be tried if desired. In this experiment 0.2 c.c. of either *ethyl butyrate* or *amyl acetate* may be employed.

IV. INVERTASES.[3]

1. **Preparation of an Extract of Sucrase.**[4]—Treat the finely divided epithelium of the small intestine of a *dog*, *pig*, *rat*, *rabbit*, or *hen* with about three volumes of a two per cent solution of sodium fluoride and permit the mixture to stand at room temperature for twenty-four hours. Strain the extract through cloth or absorbent cotton and use the strained material in the following demonstration.

2. **Demonstration of Sucrase.**—To about 5 c.c. of a one per cent solution of sucrose, in a test-tube, add about one cubic centimeter of a two per cent sodium fluoride intestinal extract, prepared

[1] A. E. Taylor: *On Fermentation; University of California Publications*, 1907.

[2] The shells should be removed without the use of water. These beans are *poisonous*, due to their content of *ricin*.

[3] *The inverting enzymes of the alimentary tract;* Mendel and Mitchell: *American Journal of Physiology*, 1907–08, XX, p. 81.

[4] For a discussion of this enzyme see p. 140.

as described above. Prepare a control tube in which the intestinal extract is *boiled* before being added to the sugar solution. Place the two tubes at 38° C. for two hours.[1] Heat the mixture to boiling to coagulate the protein material, filter, and test the filtrate by Fehling's test (see p. 27). The tube containing the *boiled* extract should give no response to Fehling's test, whereas the tube containing the *unboiled* extract should reduce the Fehling's solution. This reduction is due to the formation of *invert sugar* (see p. 41) from the sucrose through the action of the enzyme *sucrase* which is present in the intestinal epithelium.

3. **Preparation of Vegetable Sucrase.**—Thoroughly grind about 100 grams of brewer's yeast in a mortar with sand. Spread the ground yeast in thin layers on glass or porous plates and dry it rapidly in a current of dry, warm air. Powder this dry yeast, extract it with distilled water and filter. Pour the filtrate into acetone, stir and after permitting the acetone mixture to stand for a few minutes filter on a Buchner funnel. The resulting precipitate, after drying and pulverizing, may be used to demonstrate vegetable sucrase.

4. **Demonstration of Vegetable Sucrase.**—To about 5 c.c of a one per cent solution of sucrose in a test-tube add a small amount of the sucrase powder prepared as directed above. Place the tube at 38° C. for 24–72 hours and at the end of that period test the solution by Fehling's test. Reduction indicates that the active sucrase powder has transformed the non-reducing sucrose into dextrose and lævulose, and these sugars, in turn, have reduced the Fehling solution.

5. **Preparation of an Extract of Lactase.**[2]—Treat the finely divided epithelium of the small intestine of a *kitten, puppy,* or *pig embryo* with about three volumes of a two per cent solution of sodium fluoride and permit the mixture to stand at room temperature for twenty-four hours. Strain the extract through cloth or absorbent cotton and use the strained material in the following demonstration.

6. **Demonstration of Lactase.**[3]—To about 5 c.c. of a one per cent solution of lactose in a test-tube add about one cubic centimeter of a toluene-water or a two per cent sodium fluoride extract of the first part of the small intestine[4] of a *kitten, puppy,* or *pig embryo* prepared as described above. Prepare a control tube in which the intestinal extract is *boiled* before being added to the sugar solution. Place the two tubes at 38° C. for 24 hours. At the end of this period add one

[1] If a positive result is not obained in this time permit the digestion to proceed for a longer period.

[2] For a discussion of this enzyme see p. 140.

[3] Roaf; *Bio-Chemical Journal,* 1908, III, p. 182.

[4] Duodenum and first part of jejunum.

cubic centimeter of the digestion mixture to 5 c.c. of Barfoed's[1] reagent and place the tubes in a boiling water-bath.[2] Examine the tubes at the end of three minutes against a black background in a good light. If no cuprous oxide is visible replace the tubes and repeat the examination at the end of the *fourth* and *fifth* minutes. If no reduction is then observed permit the tubes to stand at room temperature for 5–10 minutes and examine again.[3]

It has been determined that disaccharide solutions will not reduce Barfoed's reagent until after they have been heated for 9–10 minutes on a boiling water-bath in contact with the reagent.[4] Therefore in the above test, if the tube containing the *unboiled* extract exhibits any reduction after being heated as indicated, for a period of five minutes or less, and the control tube containing *boiled* extract shows no reduction, it may be concluded that *lactase* was present in the intestinal extract.[5]

7. **Preparation of an Extract of Maltase.**[6]—Treat the finely divided epithelium of the small intestine of a *cat*, *kitten*, or *pig* (*embryo* or *adult*) with about three volumes of a two per cent solution of sodium fluoride and permit the mixture to stand at room temperature for twenty-four hours. Strain the extract through cloth and use the strained material in the following demonstration.

8. **Demonstration of Maltase.**—Proceed exactly as indicated in the demonstration of lactase, above, except that a one per cent solution of maltose is substituted for the lactose solution. The extract used may be prepared from the upper part of the intestine of a *cat*, *kitten*, or *pig* (*embryo* or *adult*). In the case of lactase, as indicated, the intestine used should be that of a *kitten*, *puppy*, or *pig* (*embryo*).

V. EREPSIN.[7]

1. **Preparation of Erepsin.**—Grind the mucous membrane of the small intestine of a cat, dog, or pig with sand in a mortar. Treat the mortared membrane with toluene- or chloroform-water and permit the mixture to stand, with occasional shaking, for 24–72 hours.[8]

[1] To 4.5 grams of neutral crystallized cupric acetate in 900 c.c. of water, add 0.6 c.c. of glacial acetic acid and make the total volume of the solution one liter.

[2] Care should be taken to see that the water in the bath reaches at least to the upper level of the contents of the tubes.

[3] Sometimes the drawing of conclusions is facilitated by pouring the mixture from the tube and examining the bottom of the tube for adherent cuprous oxide.

[4] The heating for 9–10 minutes is sufficient to transform the disaccharide into monosaccharide.

[5] The reduction would, of course, be due to the action of the *dextrose* and *galactose* which had been formed from the lactose through the action of the enzyme *lactase*.

[6] For a discussion of this enzyme see p. 54.

[7] Also called erepsase. For a discussion of this enzyme see p. 140.

[8] The enzyme may also be extracted by means of *glycerol* or *alkaline "physiological" salt solution* if desired.

Filter the extract thus prepared through cotton and use the filtrate in the following experiment.

2. **Demonstration of Erepsin.**—To about 5 c.c. of a one per cent solution of Witte's peptone in a test-tube add about one c.c. of the erepsin extract prepared as described above and make the mixture slightly alkaline (0.1 per cent) with sodium carbonate. Prepare a second tube containing a like amount of peptone solution but *boil* the erepsin extract before introducing it. Place the two tubes at 38° C. for 2–3 days. At the end of that period heat the contents of each tube to boiling, filter and try the biuret test on each filtrate. In making these tests care should be taken to use like amounts of filtrate, potassium hydroxide and cupric sulphate in each test in order that the drawing of correct conclusions may be facilitated. The contents of the tube which contained the *boiled* extract should show a deep pink color with the biuret test, due to the peptone still present. On the other hand, the biuret test upon the contents of the tube containing the *unboiled* extract should be negative or exhibit, at the most, a *faint pink* or *blue color*, signifying that the peptone, through the influence of the erepsin, has been transformed, in great part at least, into *amino acids* which do not respond to the biuret test.[1]

VI. URICOLYTIC ENZYME.[2]

1. **Preparation of Uricolytic Enzyme.**—Extract pulped liver tissue with toluene- or chloroform-water at 38° C. for 24 hours, with occasional shaking. Filter the extract and use the filtrate in the following experiment.

2. **Demonstration of Uricolytic Enzyme.**—Add about 0.1 gram of uric acid to 10 c.c. of water and bring the uric acid into solution by the addition of the minimal quantity of potassium hydroxide. To 5 c.c. of this uric acid solution, in a test-tube, add 5 c.c. of the uricolytic enzyme extract prepared as described above. Prepare a second tube containing a like amount of uric acid solution, but *boil* the extract before it is introduced. Place the two tubes at 38° C. for 3–4 days and titrate the two digestive mixtures with a solution of potassium permanganate according to directions given under Folin-Shaffer Method, Chapter XXII. It will be found that the mixture containing the *boiled* extract requires a much larger volume of the

[1] *Strictly speaking*, this erepsin demonstration is not adequate unless a control test is made with *native protein* (except caseinogen, histones and protamines) to show that the extract is *trypsin-free* and digests peptone but not native protein.

[2] Mendel and Mitchell; *American Journal of Physiology*, 1908, XX, p. 97.

permanganate to complete the titration than the other tube. This indicates that a uricolytic enzyme has destroyed at least a portion of the uric acid which was originally present in the tube containing the *unboiled* extract.

VII. CATALASE.

Demonstration of Catalase.—The various animal tissues, such as *liver*, *kidney*, *blood*, *lung*, *muscle* and *brain*, contain an enzyme called *catalase* which possesses the property of decomposing hydrogen peroxide. The presence of this enzyme may be demonstrated as follows: Introduce into a low, broad, wide-mouthed bottle some pulped liver tissue and a porcelain crucible containing *neutral* hydrogen peroxide.[1] Connect the bottle with a eudiometer filled with water, upset the crucible of hydrogen peroxide upon the liver pulp and note the collection of gas in the eudiometer. This gas is oxygen which has been liberated from the hydrogen peroxide through the action of the catalase of the liver tissue.

B. Experiments on Anti-Enzymes.

1. **Preparation of an Extract of Anti-Pepsin.**[2]—Grind up a number of intestinal worms (ascaris)[3] with quartz sand in a mortar. Subject this mass to high pressure, filter the resultant juice and treat it with alcohol until a concentration of sixty per cent is reached. If any precipitate forms it should be filtered off[4] and alcohol added to the filtrate until the concentration of alcohol is 85 per cent, or over. The anti-enzyme is precipitated by this concentration. Permit this precipitate to stand for twenty-four hours, then filter it off, wash it with 95 per cent alcohol, absolute alcohol, and ether, in turn, and finally dry the substance over sulphuric acid. The sticky powder which results may be used in this form or may be dissolved in water as desired and the aqueous solution used.[5]

2. **Demonstration of Anti-Pepsin.**[6]—Introduce into a test-tube a few fibrin shreds and equal volumes of pepsin-hydrochloric acid[7] and ascaris extract made as indicated above. Prepare a control tube in which the ascaris extract is replaced by water. Place the

[1] Mendel and Leavenworth; *American Journal of Physiology*, 1908, XXI, p. 85.

[2] Anti-gastric-protease or anti-acid-protease.

[3] These may be readily obtained from pigs at a slaughter house.

[4] This precipitate consists of impurities, the anti-enzyme not being precipitated until a higher concentration of alcohol is reached.

[5] The original ascaris extract possesses much greater activity than either the powder or the aqueous solution.

[6] Martin H. Fischer; *Physiology of Alimentation*, 1907, p. 134.

[7] Made by bringing 0.015 gram of pepsin into solution in 7 c.c. of water and 0.23 gram of concentrated hydrochloric acid.

tubes at 38° C. Ordinarily in one hour the fibrin in the control tube will be completely digested. The fibrin in the tube containing the ascaris extract may, however, remain unchanged for days, thus indicating the inhibitory influence exerted by the anti-enzyme present in this extract.

3. **Preparation of an Extract of Anti-Trypsin.**[1]—The extract may be prepared from the intestinal worm, ascaris, according to the directions given on page 14.

4. **Demonstration of Anti-Trypsin.**—Introduce into a test-tube a few shreds of fibrin and equal volumes of an artificial tryptic solution[2] and the ascaris extract made as described on page 14. Prepare a control tube in which the ascaris extract is replaced by water. Place the two tubes at 38° C. Ordinarily the fibrin in the control tube will be completely digested in two hours. The fibrin in the tube containing the ascaris extract may, however, remain unchanged for days, thus indicating the inhibitory influence of the anti-enzyme.

Blood serum also contains *anti-trypsin*. This may be demonstrated as follows: Introduce equal volumes of serum and artificial tryptic solution (prepared as described above) into a test-tube and add a few shreds of fibrin. Prepare a *control* tube containing *boiled* serum. Place the two tubes at 38° C. It will be observed that the fibrin in the tube containing the *boiled* serum digests, whereas that in the other tube does not digest. The anti-trypsin present in the unboiled serum has exerted an inhibitory influence upon the action of the trypsin.

C. Quantitative Applications.

1. **Quantitative Determination of Amylolytic Activity.**—*Wohlgemuth's Method.*—Arrange a series of test-tubes with diminishing quantities of the enzyme solution under examination, introduce into each tube 5 c.c. of a 1 per cent solution of soluble starch[3] and place each tube at once in a bath of ice-water.[4] When all the tubes have

[1] Anti-pancreatic-protease or anti-alkali-protease.

[2] Made by dissolving 0.04 gram of sodium carbonate and 0.015 gram of trypsin in 8 c.c. of water.

[3] Kahlbaum's soluble starch is satisfactory. In preparing the 1 per cent solution, the weighed starch powder should be dissolved in the proper volume of *cold* distilled water and stirred until a homogeneous suspension is obtained. The mixture should then be heated, with constant stirring, in a porcelain dish, until it is clear. This ordinarily takes about 8–10 minutes. A slightly opaque solution is thus obtained which should be cooled before using.

[4] Ordinarily a series of six tubes is satisfactory, the volumes of the enzyme solution used ranging from 1 c.c. to 0.1 c.c. and the measurements being made by means of a 1 c.c. graduated pipette. Each tube should be paced in the ice-water bath as soon as the starch solution is introduced. It will be found convenient to use a small wire basket to hold the tubes.

been prepared in this way and placed in the ice-water bath they are transferred to a water-bath or incubator and kept at 38° C. for from thirty minutes to an hour.[1] At the end of this digestion period the tubes are again removed to the bath of ice water in order that the action of the enzyme may be stopped.

Dilute the contents of each tube, to within about one-half inch of the top, with water, add one drop of a N/10 solution of iodine and shake the tube and contents thoroughly. A series of colors ranging from *dark blue* through *bluish-violet* and *reddish-yellow* to *yellow*, will be formed.[2] The dark blue color shows the presence of unchanged starch, the bluish-violet indicates a mixture of starch and erythrodextrin, whereas the reddish-yellow signifies that erythrodextrin and maltose are present, and the yellow solution denotes the complete transformation of starch into maltose. Examine the tubes carefully before a white background and select the last tube in the series which shows the entire absence of all blue color, thus indicating that the starch has been completely transformed into dextrins and sugar. In case of indecision between two tubes, add an extra drop of the iodine solution, and observe them again, after shaking.

Calculation.—The amylolytic activity[3] of a given solution is expressed in terms of the activity of 1 c.c. of such a solution. For example, if it is found that 0.02 c.c. of an amylolytic solution, acting at 38° C., completely transformed the starch in 5 c.c. of a 1 per cent starch solution in 30 minutes, the amylolytic activity of such a solution would be expressed as follows:

$$D^{38°}_{30'} = 250.$$

This indicates that 1 c.c. of the solution under examination possesses the power of completely digesting 250 c.c. of a 1 per cent starch solution in 30 minutes at 38° C.

2. **Quantitative Determination of Peptic Activity.**—(*a*) *Mett's Method.*—The determination of the actual rate of peptic activity is a most important procedure under certain conditions. Several methods of making this determination are in use. The method of Sprigg[4] is probably the most accurate method yet devised for this purpose. It is, however, too complicated and time-consuming for clinical

[1] Longer digestion periods may be used where it is deemed advisable. If exceedingly weak solutions are being investigated, it may be most satisfactory to permit the digestion to extend over a period of 24 hours.

[2] See p. 45.

[3] Designated by "D" the first letter of "diastatic."

[4] Sprigg: *Zeitschrift für physiologische Chemie*, 1902, XXXV, p. 465.

purposes. The method of Mett, given below, is very simple although not strictly accurate. The procedure is as follows: To about 5 c.c. of the gastric juice under examination in a test-tube add a section of a Mett tube[1] and place the mixture at 38° C. for *ten hours*. At the end of this period, the tube should be removed from the gastric juice and the length of the column of coagulated albumin which has been digested carefully determined by means of a low-power microscope and a millimeter scale. In normal human gastric juice the upper limit is 4 mm. However, control tests should always be made to determine the digestibility of the coagulated albumin in artificial gastric juice, inasmuch as this factor will vary with different albumin preparations.

In connection with this test Schütz's law should be borne in mind. This principle is to the effect that *the amount of proteolytic enzyme present in a digestion mixture is proportional to the square of the number of millimeters of albumin digested.* Therefore a gastric juice which digests 2 mm. of albumin contains *four times* as much pepsin as a gastric juice which digests only 1 mm. of albumin. And further, if the quantities of albumin digested are 2 mm. and 3 mm., respectively, the ratio between the pepsin values will be as 4:9.

It is claimed by Nirenstein and Schiff that the principle of Schütz does not apply to gastric juice unless this fluid be diluted with fifteen volumes of N/20 hydrochloric acid.

(*b*) *Fuld and Levison's Method.*—This test is founded upon the fact, shown by Osborne, that edestin when brought into solution in dilute acid will change in its solubility, due to the contact with the acid, and that a protean called *edestan*, which is insoluble in neutral fluid, will be formed. The procedure is as follows: Dilute the gastric juice under examination with 20 volumes of water and introduce gradually decreasing volumes of the diluted juice into a series[2] of narrow test-tubes about 1 cm. in diameter. The measurements of gastric juice may conveniently be made with a one c.c. pipette which is accurately graduated in 1/100 c.c. Into the first tube in the series may be introduced one c.c. of gastric juice, and the tubes which

[1] In the preparation of these tubes, egg-white is diluted with an equal volume of water, the precipitated globulin filtered off and the filtrate collected in a tall, narrow beaker or a large test-tube. A bundle of capillary tubes about 10 cm. in length and 2 mm. in diameter are now placed in this vessel in such a manner that they are completely submerged in the albumin solution. After an examination has shown that the tubes are completely filled with the albumin solution and that there are no interfering *air-bubbles*, the vessel and its contained tubes is heated for 5–15 minutes in a boiling water-bath, in order to coagulate the albumin. When this coagulation is complete, the tubes are removed, all albumin adhering to them is carefully cleaned off, and the tubes rendered air-tight by the application of sealing wax at either end. When needed for use, these tubes are cut into sections about 2 cm. in length.

[2] The longer the series, the more accurate the deductions which may be drawn.

follow in the series may receive volumes which differ, in each instance, from the volume introduced into the preceding tube by 1/100, 1/50, 1/20, or 1/10 of a cubic centimeter. Now *rapidly* introduce into each tube the same volume (*e. g.*, 2 c.c.) of a 1:1000 solution of *edestin*[1] and place the tubes at 40° C. for one-half hour. At the end of this time stratify ammonium hydroxide upon the contents of each tube,[2] place the tubes in position before a black background and examine them carefully. The ammonium hydroxide, by diffusing into the acid fluid, forms a neutral zone and in this zone will be precipitated any undigested *edestan* which is present. Select the tube in the series which contains the least amount of gastric juice and which *exhibits no ring*, signifying that the *edestan* has been *completely digested*, and calculate the peptic activity of the gastric juice under examination on the basis of the volume of gastric juice used in this particular tube.

Calculation.—Multiply the number of c.c. of edestin solution used by the dilution to which the gastric juice was originally subjected and divide the volume of gastric juice necessary to completely digest the edestan by this product. For example, if 2 c.c. of the edestin solution was completely digested by 0.25 c.c. of a 1:20 gastric juice we would have the following expression: $0.25 \div 20 \times 2$ or 1:160. This peptic activity may be expressed in several ways, *e. g.*, (*a*) 1:160 pepsin; (*b*) 160 pepsin content; (*c*) 160 parts.

3. **Quantitative Determination of Tryptic Activity.**—*Gross' Method.*—This method is based upon the principle that faintly alkaline solutions of casein are precipitated upon the addition of dilute (1 per cent) acetic acid whereas its digestion products are not so precipitated. The method follows: Prepare a series of tubes each containing 10 c.c. of a 0.1 per cent solution of pure, fat-free casein,[3] which has been heated to a temperature of 40° C. Add to the contents of the series of tubes increasing amounts of the trypsin solution under examination,[4] and place them at 40° C. for *fifteen minutes*. At the end of this time remove the tubes and acidify the contents of each with a few drops of dilute (1 per cent) acetic acid. The tubes

[1] This edestin should be prepared in the usual way (see p. 101), and brought into solution in a dilute hydrochloric acid of approximately the same strength as that which occurs normally in the human stomach. This may be conveniently made by adding 30 c.c. of N/10 hydrochloric acid to 70 c.c. of water. Ordinarily it should not take longer than *one minute* to introduce the edestin solution into the entire series of tubes. However, if the edestin is added to the tubes in the same order as the ammonium hydroxide is afterward stratified, no appreciable error is introduced.

[2] Making the stratification in the *same order* as the edestin solution was added.

[3] Made by dissolving one gram of Grübler's casein in a liter of 0.1 per cent sodium carbonate. A little chloroform may be added to prevent bacterial action.

[4] The amount of solution used may vary from 0.1–1 c.c. The measurements may conveniently be made by means of a 1 c.c. graduated pipette.

in which the casein is completely digested will remain clear when acidified, while those tubes which contain *undigested* casein will become more or less *turbid* under these conditions. Select the first tube in the series which exhibits *no turbidity* upon acidification, thus indicating complete digestion of the casein, and calculate the tryptic activity of the enzyme solution under examination.

Calculation.—The *unit* of tryptic activity is an expression of the power of 1 c.c. of the fluid under examination exerted for a period of fifteen minutes on 10 c.c. of a 0.1 per cent casein solution. For example, if 0.5 c.c. of a trypsin solution completely digests 10 c.c. of a 0.1 per cent solution of casein in fifteen minutes the activity of that solution would be expressed as follows:

$$\text{Tryptic activity} = 1 \div 0.5 = 2.$$

Such a trypsin solution would be said to possess an activity of 2. If 0.3 c.c. of the trypsin solution had been required the solution would be said to possess an activity of 3.3; *i. e.*, $1 \div 0.3 = 3.3$.

CHAPTER II.

CARBOHYDRATES.

THE name carbohydrates is given to a class of bodies which are an especially prominent constituent of plants and which are found also in the animal body either free or as an integral part of various proteins. They are called carbohydrates because they contain the elements C, H and O; the H and O being present in the proportion to form water. The term is not strictly appropriate inasmuch as there are bodies, such as acetic acid, lactic acid and inosite, which have H and O present in the proportion to form water, but which are not carbohydrates, and there are also true carbohydrates which do not have H and O present in this proportion, *e. g.*, rhamnose, $C_6H_{12}O_5$.

Chemically considered, the carbohydrates are aldehyde or ketone derivatives of complex alcohols. Treated from this standpoint, the aldehyde derivatives are spoken of as aldoses, and the ketone derivatives are spoken of as ketoses. The carbohydrates are also frequently named according to the number of oxygen atoms present in the molecule, *e. g.*, trioses, pentoses, and hexoses.

The more common carbohydrates may be classified as follows:

I. Monosaccharides.
   1. Hexoses, $C_6H_{12}O_6$.
      (*a*) Dextrose.
      (*b*) Lævulose.
      (*c*) Galactose.
   2. Pentoses, $C_5H_{10}O_5$.
      (*a*) Arabinose.
      (*b*) Xylose.
      (*c*) Rhamnose (Methyl-pentose), $C_6H_{12}O_5$.

II. Disaccharides, $C_{12}H_{22}O_{11}$.
   1. Maltose.
   2. Lactose.
   3. Iso-Maltose.
   4. Sucrose.

III. Trisaccharides, $C_{18}H_{32}O_{16}$.
   1. Raffinose.
IV. Polysaccharides, $(C_6H_{10}O_5)_x$.
   1. Starch Group.
      (*a*) Starch.
      (*b*) Inulin.
      (*c*) Glycogen.
      (*d*) Lichenin.
   2. Gum and Vegetable Mucilage Group.
      (*a*) Dextrin.
      (*b*) Vegetable Gums.
   3. Cellulose Group.
      (*a*) Cellulose.
      (*b*) Hemi-Cellulose.

Each member of the above carbohydrate classes, except the members of the pentose group, may be supposed to contain the group $C_6H_{10}O_5$, called the *saccharide group*. The polysaccharides consist of this group alone taken a large number of times, whereas the disaccharides may be supposed to contain two such groups plus a molecule of water, and the monosaccharides to contain one such group plus a molecule of water. Thus, $(C_6H_{10}O_5)_x$ = polysaccharide, $(C_6H_{10}O_5)_2 + H_2O$ = disaccharide, $C_6H_{10}O_5 + H_2O$ = monosaccharide. In a general way the solubility of the carbohydrates varies with the number of saccharide groups present, the substances containing the largest number of these groups being the least soluble. This means simply that, as a class, the monosaccharides (hexoses) are the most soluble and the polysaccharides (starches and cellulose) are the least soluble.

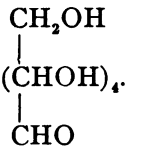
MONOSACCHARIDES.

Hexoses, $C_6H_{12}O_6$.

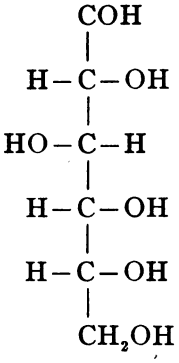
The hexoses are monosaccharides containing six oxygen atoms in a molecule. They are the most important of the simple sugars, and two of the principal hexoses, dextrose and lævulose, occur widely distributed in plants and fruits. Of these two hexoses, dextrose results from the hydrolysis of starch whereas both dextrose and lævulose are formed in the hydrolysis of sucrose. Galactose, which with dextrose results from the hydrolysis of lactose, is also an important hexose. These three hexoses are fermentable by yeast, and yield

lævulinic acid upon heating with dilute mineral acids. They reduce metallic oxides in alkaline solution, are optically active and extremely soluble. With phenylhydrazine they form characteristic osazones.

DEXTROSE,



Dextrose, also called glucose or grape sugar, is present in the blood in small amount and may also occur in traces in normal urine. After the ingestion of large amounts of sucrose, lactose or dextrose, causing the *assimilation limit* to be exceeded, an alimentary glycosuria may arise. In diabetes mellitus very large amounts of dextrose are excreted in the urine. The following structural formula has been suggested by Victor Meyer for *d*-dextrose:



(For further discussion of dextrose see section on Hexoses, page 21.)

Experiments on Dextrose.

1. **Solubility.**—Test the solubility of dextrose in the "ordinary solvents" and in alcohol. (In the solubility tests throughout the book we shall designate the following solvents as the "ordinary solvents": H_2O; 10 per cent NaCl; 0.5 per cent Na_2CO_3; 0.2 per cent HCl; concentrated KOH; concentrated HCl.)

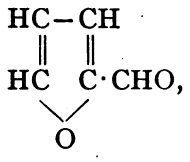
2. **Molisch's Reaction.**—Place approximately 5 c.c. of concentrated H_2SO_4 in a test-tube. Incline the tube and slowly pour down the inner side of it approximately 5 c.c. of the sugar solution to which 2 drops of Molisch's reagent (a 15 per cent alcoholic solution of α-naph-

PLATE III.

OSAZONS.

Upper form, dextrosazon; central form, maltosazon; lower form, lactosazon.

thol) has been added, so that the sugar solution will not mix with the acid. A reddish-violet zone is produced at the point of contact. The reaction is due to the formation of furfurol,



by the acid. The test is given by all bodies containing a carbohydrate group and is therefore not specific and, in consequence, of very little practical importance.

3. **Phenylhydrazine Reaction.**—Test according to one of the following methods: (*a*) To a small amount of phenylhydrazine mixture, furnished by the instructor,[1] add 5 c.c. of the sugar solution, shake well and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the tube to cool *slowly* and examine the crystals microscopically (Plate III, opposite). If the solution has become too concentrated in the boiling process it will be light red in color and no crystals will separate until it is diluted with water.

Yellow crystalline bodies called *osazones* are formed from certain sugars under these conditions, in general each individual sugar giving rise to an osazone of a definite crystalline form which is typical for that sugar. It is important to remember in this connection that of the simple sugars of interest in physiological chemistry, dextrose and lævulose yield the same osazone. Each osazone has a definite melting-point and as a further and more accurate means of identification it may be recrystallized and identified by the determination of its melting-point and nitrogen content. The reaction taking place in the formation of *phenyldextrosazone* is as follows:

$$\underset{\text{Dextrose.}}{C_6H_{12}O_6} + 2\underset{\text{Phenylhydrazine.}}{(H_2N\cdot NH\cdot C_6H_5)} = \underset{\text{Phenyldextrosazone.}}{C_6H_{10}O_4(N\cdot NH\cdot C_6H_5)_2} + 2H_2O + H_2.$$

(*b*) Place 5 c.c. of the sugar solution in a test-tube, add 1 c.c of the phenylhydrazine-acetate solution furnished by the instructor,[2] and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the liquid to cool *slowly* and examine the crystals microscopically (Plate III, opposite).

[1] This mixture is prepared by combining one part of phenylhydrazine hydrochloride and two parts of sodium acetate, *by weight*. These are thoroughly mixed in a mortar.

[2] This solution is prepared by mixing one part *by volume*, in each case, of glacial acetic acid, one part of water and two parts of phenylhydrazine (the base).

The phenylhydrazine test has been so modified by Cipollina as to be of use as a *rapid clinical test.* The directions for this test are given in the next experiment.

4. **Cipollina's Test.**—Thoroughly mix 4 c.c. of dextrose solution, 5 drops of phenylhydrazine (the base) and 1/2 c.c. of glacial acetic acid in a test-tube. Heat the mixture for about one minute over a low flame, shaking the tube continually to prevent loss of fluid by bumping. Add 4–5 drops of sodium hydroxide (sp. gr. 1.16), being certain that the fluid in the test-tube remains acid, heat the mixture again for a moment and then cool the contents of the tube. Ordinarily the crystals form at once, especially if the sugar solution possesses a low specific gravity. If they do not appear immediately allow the tube to stand at least 20 minutes before deciding upon the absence of sugar.

Examine the crystals under the microscope and compare them with those shown in Plate III, opposite page 23.

5. **Riegler's Reaction.**[1]—Introduce 0.1 gram of phenylhydrazine-hydrochloride and 0.25 gram of sodium acetate into a test-tube, add 20 drops of the solution under examination and heat the mixture to boiling. Now introduce 10 c.c. of a 3 per cent solution of potassium hydroxide and gently shake the tube and contents. If the solution under examination contains dextrose the liquid in the tube will assume a red color. One per cent dextrose yields an immediate color whereas 0.05 per cent yields the color only after the lapse of a period of one-half hour from the time the alkali is added. In urinary examination if the color appears after the thirty-minute interval the color change is without significance inasmuch as sugar-free urine will respond thus. The reaction is given by all aldehydes and therefore the test cannot be safely employed in testing urines preserved by formaldehyde. Albumin does not interfere with the test.

6. **Bottu's Test.**[2]—To 8 c.c. of Bottu's reagent[3] in a test-tube add 1 c.c. of the solution under examination and mix the liquids by gentle shaking. Now heat the upper portion of the mixture to boiling, add an additional 1 c.c. of the solution and heat the mixture again immediately. The appearance of a blue color accompanied by the precipitation of small particles of indigo blue indicates the presence of dextrose in the solution under examination. The test will serve to detect the presence of 0.1 per cent of dextrose.

[1] Riegler; Compt. rend. soc. biol., 66, p. 795.
[2] Bottu; Compt. rend. soc. biol., 66, p. 972.
[3] This reagent contains 3.5 grams of *o*-nitrophenylpropiolic acid and 5 c.c. of a freshly prepared 10 per cent solution of sodium hydroxide per liter.

7. **Precipitation by Alcohol.**—To 10 c.c. of 95 per cent alcohol add about 2 c.c. of dextrose solution. Compare the result with that obtained under Dextrin, 7, page 48.

8. **Iodine Test.**—Make the regular iodine test as given under Starch, 5, page 45, and keep this result in mind for comparison with the results obtained later with starch and with dextrin.

9. **Diffusibility of Dextrose.**—Test the diffusibility of dextrose solution through animal membrane, or parchment paper, making a dialyzer like one of the models shown in Fig. 1.

10. **Moore's Test.**—To 2–3 c.c. of sugar solution in a test-tube add an equal volume of concentrated KOH or NaOH, and boil. The solution darkens and finally assumes a brown color. At this point the

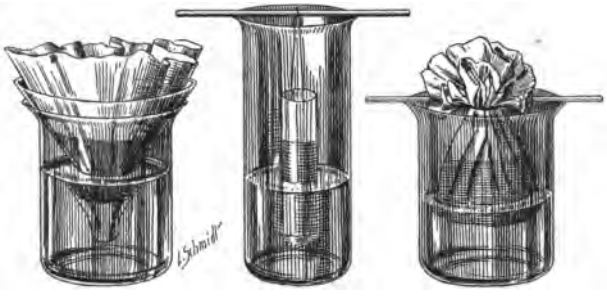
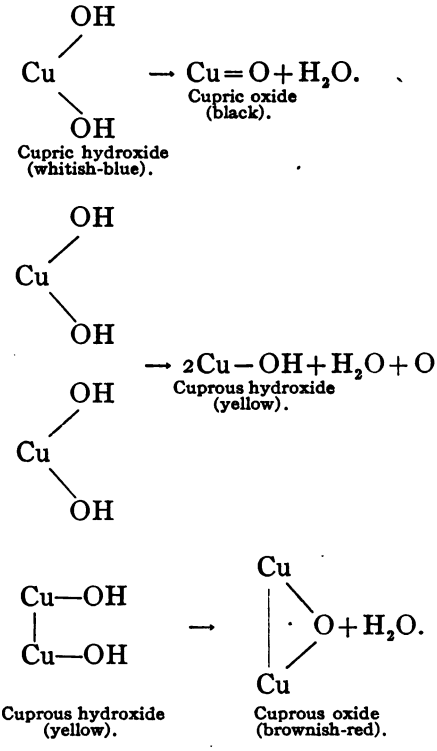


FIG. 1.—DIALYZING APPARATUS FOR STUDENTS' USE.

odor of caramel may be detected. This is an exceedingly crude test and is of little practical value. The brown color is due to the oxidation of the dextrose and the resulting formation of the potassium or sodium salts of certain organic acids which are formed as oxidation products.

11. **Reduction Tests.**—To their aldehyde or ketone structure many sugars owe the property of readily reducing alkaline solutions of the oxides of metals like copper, bismuth and mercury; they also possess the property of reducing ammoniacal silver solutions with the separation of metallic silver. Upon this property of reduction the most widely used tests for sugars are based. When whitish-blue cupric hydroxide in suspension in an alkaline liquid is heated it is converted into insoluble black cupric oxide, but if a reducing agent like certain sugars be present the cupric hydroxide is reduced to insoluble yellow cuprous hydroxide, which in turn, on further heating, may be

converted into brownish-red or red cuprous oxide. These changes are indicated as follows:



The chemical equations here discussed are exemplified in Trommer's and Fehling's tests.

(*a*) *Trommer's Test.*—To 5 c.c. of sugar solution in a test-tube add one-half its volume of KOH or NaOH. Mix thoroughly and add, drop by drop, a *very dilute* solution of cupric sulphate. Continue the addition until there is a slight permanent precipitate of cupric hydroxide and in consequence the solution is slightly turbid. Heat, and the cupric hydroxide is reduced to yellow cuprous hydroxide or to brownish-red cuprous oxide. If the solution of cupric sulphate used is too strong a small brownish-red precipitate produced in a weak sugar solution may be entirely masked. On the other hand, particularly in testing for sugar in the urine, if too little cupric sulphate is used a light-colored precipitate formed by uric acid and purine bases may obscure the brownish-red precipitate of cuprous oxide. The action of KOH or NaOH in the presence of an excess of sugar and insufficient copper will produce a brownish color. Phosphates

of the alkaline earths may also be precipitated in the alkaline solution and be mistaken for cuprous hydroxide. Trommer's test is not very satisfactory.

(*b*) *Fehling's Test.*—To about 1 c.c. of Fehling's solution[1] in a test-tube add about 4 c.c. of water, and boil. This is done to determine whether the solution will of itself cause the formation of a precipitate of brownish-red cuprous oxide. If such a precipitate forms, the Fehling's solution must not be used. Add sugar solution to the warm Fehling's solution *a few drops* at a time and heat the mixture after each addition. The production of yellow cuprous hydroxide or brownish-red cuprous oxide indicates that reduction has taken place. The yellow precipitate is more likely to occur if the sugar solution is added rapidly and in large amount, whereas with a less rapid addition of smaller amounts of sugar solution the brownish-red precipitate is generally formed.

This is a much more satisfactory test than Trommer's, but even this test is not entirely reliable when used to detect sugar in the urine. Such bodies as *conjugate glycuronates*, *uric acid*, *nucleo-protein* and *homogentisic acid* when present in sufficient amount may produce a result similar to that produced by sugar. *Phosphates of the alkaline earths* may be precipitated by the alkali of the Fehling's solution and in appearance may be mistaken for cuprous hydroxide. Cupic hydroxide may also be reduced to cuprous oxide and this in turn be dissolved by *creatinine*, a normal urinary constituent. This will give the urine under examination a greenish tinge and may obscure the sugar reaction even when a considerable amount of sugar is present.

(*c*) *Benedict's Modifications of Fehling's Test.—First Modification.*—To 2 c.c. of Benedict's solution[2] in a test-tube add 6 c.c. of distilled water and 7–9 drops (not more) of the solution under examination. Boil the mixture vigorously for about 15–30 seconds and permit it

[1] Fehling's solution is composed of two definite solutions—a cupric sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

Cupric sulphate solution = 34.65 grams of cupric sulphate dissolved in water and made up to 500 c.c.

Alkaline tartrate solution = 125 grams of potassium hydroxide and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

[2] Benedict's modified Fehling solution consists of two definite solutions—a cupric sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

Cupric sulphate solution = 34.65 grams of cupric sulphate dissolved in water and made up to 500 c.c.

Alkaline tartrate solution = 100 grams of anhydrous *sodium carbonate* and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

to cool to room temperature spontaneously. (If desired this process may be repeated, although it is ordinarily unnecessary.) If sugar is present in the solution a precipitate will form which is often *bluish-green* or *green* at first, especially if the percentage of sugar is low, and which usually becomes *yellowish* upon standing. If the sugar present exceeds 0.06 per cent this precipitate generally forms at or below the boiling point, whereas if less than 0.06 per cent of sugar is present the precipitate forms more slowly and generally only after the solution has cooled.

Benedict claims, whereas the original Fehling test will not serve to detect sugar when present in a concentration of less than 0.1 per cent, that the above modification will serve to detect sugar when present in as small quantity as 0.015–0.02 per cent.

The modified Fehling solution used in the above test differs from the original Fehling solution in that 100 grams of sodium carbonate is substituted for the 125 grams of potassium hydroxide ordinarily used, thus forming a Fehling solution which is considerably less alkaline than the original. This alteration in the composition of the Fehling solution is of advantage in the detection of sugar in the urine inasmuch as the strong alkalinity of the ordinary Fehling solution has a tendency, when the reagent is boiled with a urine containing a small amount of dextrose, to decompose sufficient of the sugar to render the detection of the remaining portion exceedingly difficult by the usual technic. Benedict claims that for this reason the use of his modified solution permits the detection of much smaller amounts of sugar than does the use of the ordinary Fehling solution. He has further modified his solution for use in the quantitative determination of sugar (see Chapter XXII).

Second Modification.[1]—Very recently Benedict has further modified his solution and has succeeded in obtaining one which does not deteriorate upon long standing.[2] The following is the procedure for the detection of dextrose in solution: To five cubic centimeters of the reagent in a test-tube add eight (not more) drops of the solu-

[1] Private communication from Dr. S. R. Benedict.

[2] Benedict's new solution has the following composition:

Cupric sulphate	17.3 grams.
Sodium citrate	173.0 grams.
Sodium carbonate (anhydrous)	100.0 grams.

Distilled water to make 1 liter.

With the aid of heat dissolve the sodium citrate and carbonate in about 600 c.c. of water. Pour (through a folded filter paper if necessary) into a glass graduate and make up to 850 c.c. Dissolve the cupric sulphate in about 100 c.c. of water and make up to 150 c.c. Pour the carbonate-citrate solution into a large beaker or casserole and add the cupric sulphate solution slowly, with constant stirring. The mixed solution is ready for use and does not deteriorate upon long standing.

tion under examination. Boil the mixture vigorously for from one to two minutes and then allow the fluid to cool *spontaneously.* In the presence of dextrose *the entire body of the solution will be filled* with a precipitate, which may be *red, yellow* or *green* in color, depending upon the amount of sugar present. If no dextrose is present, the solution will remain perfectly clear. (If urine is being tested, it may show a very faint turbidity, due to precipitated urates.) Even very small quantities of dextrose (0.1 per cent) yield precipitates of surprising bulk with this reagent, and the positive reaction for dextrose is the filling of the entire body of the solution with a precipitate, so that the solution becomes opaque. Since *amount* rather than color of the precipitate is made the basis of this test, it may be applied even for the detection of small quantities of dextrose, as readily in artificial light as in daylight.

(*d*) *Boettger's Test.*—To 5 c.c. of sugar solution in a test-tube add 1 c.c. of KOH or NaOH and a very small amount of bismuth subnitrate, and boil. The solution will gradually darken and finally assume a black color due to reduced bismuth. If the test is made on urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced (bismuth sulphide).

(*e*) *Nylander's Test* (*Almén's Test*).—To 5 c.c. of sugar solution in a test-tube add one-tenth its volume of Nylander's reagent[1] and heat for five minutes in a boiling water-bath.[2] The solution will darken if reducing sugar is present, and upon standing for a few moments a black color will appear. This color is due to the precipitation of bismuth. If the test is made on urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced. Dextrose when present to the extent of 0.08 per cent may be detected by this reaction. It is claimed by Bechold that Nylander's and Boettger's tests give a negative reaction with solutions containing sugar when mercuric chloride or chloroform is present. Other observers[3] have failed to verify the inhibitory action of mercuric chloride and have shown that the inhibitory influence of chloroform may be overcome by

[1] Nylander's reagent is prepared by digesting 2 grams of bismuth subnitrate and 4 grams of Rochelle salt in 100 c.c. of a 10 per cent potassium hydroxide solution. The reagent is then cooled and filtered.

[2] Hammarsten suggests that the mixture should be boiled 2–5 minutes (according to the sugar content) over a free flame and the tube then permitted to stand 5 minutes before drawing conclusions.

[3] Rehfuss and Hawk; *Journal of Biological Chemistry*, VII, p. 267, 1910; also Zeidlitz: *Upsala Lakäreforen Forh.*, N. F., XI, 1906.

raising the temperature of the urine to the boiling point for a period of five minutes previous to making the test. Urines rich in *indican*, *urochrome*, *uroerythrin* or *hæmatoporphyrin*, as well as urines excreted after the ingestion of large amounts of certain *medicinal substances*, may give a darkening of Nylander's reagent similar to that of a true sugar reaction. It is a disputed point whether the urine after the administration of urotropin will reduce Nylander's reagent.[1]

According to Rustin and Otto, the addition of $PtCl_4$ increases the delicacy of Nylander's reaction. They claim that this procedure causes the sugar to be converted *quantitatively*. No quantitative method has yet been devised, however, based upon this principle.

Bohmansson[2] before testing the urine under examination treats it (10 c.c.) with 1/5 volume of 25 per cent hydrochloric acid and about 1/2 volume of bone black. This mixture is shaken one minute, then filtered and the neutralized filtrate tested by Nylander's reaction. Bohmansson claims that this procedure removes certain interfering substances, in particular *urochrome*.

A positive Nylander or Boettger test is probably due to the following reactions:

$$(a) \qquad Bi(OH)_2NO_3 + KOH = Bi(OH)_3 + KNO_3.$$

$$(b) \qquad 2Bi(OH)_3 - 3O = Bi_2 + 3H_2O.$$

12. **Fermentation Test.**—"Rub up" in a mortar about 20 c.c. of the sugar solution with a small piece of compressed yeast. Transfer the mixture to a saccharometer (shown in Fig. 2, p. 31) and stand it aside in a warm place for about twelve hours. If the sugar is fermentable, alcoholic fermentation will occur and carbon dioxide will collect as a gas in the upper portion of the tube. On the completion of fermentation introduce a little potassium hydroxide solution into the graduated portion by means of a bent pipette, place the thumb *tightly* over the opening in the apparatus and invert the saccharometer. Explain the result.

13. **Barfoed's Test.**—Place about 5 c.c. of Barfoed's solution[3] in a test-tube and heat to boiling. Add dextrose solution slowly, a few drops at a time, heating after each addition. Reduction is indicated by the formation of a red precipitate. If the precipitate does not

[1] Abt; *Archives of Pediatrics*, XXIV, p. 275, 1907; also Weitbrecht; *Schweiz. Wochschr.*, XLVII, p. 577, 1909.

[2] Bohmansson: *Biochem. Zeit.*, 19, p. 281.

[3] Barfoed's solution is prepared as follows: Dissolve 4.5 grams of neutral crystallized cupric acetate in 100 c.c. of water and add 1.2 c.c. of 50 per cent acetic acid.

form upon continued boiling allow the tube to stand a few minutes and examine. Sodium chloride interferes with the reaction (Welker).

Barfoed's test is *not* a specific test for dextrose as is frequently stated, but simply serves to detect *monosaccharides*. Disaccharides will also respond to the test, according to Hinkel and Sherman, if the sugar solution is boiled sufficiently long, in contact with the reagent, to hydrolyze the disaccharide through the action of the acetic acid present in the Barfoed's solution.

14. **Formation of Caramel.**—Gently heat a small amount of pulverized dextrose in a test-tube. After the sugar has melted and turned brown, allow the tube to cool, add water and warm. The coloring matter produced is known as *caramel.*

15. **Demonstration of Optical Activity.**—A demonstration of the use of the polariscope, by the instructor, each student being required to take readings and compute the "specific rotation."

FIG. 2.—EINHORN SACCHAROMETER.

USE OF THE POLARISCOPE.

For a detailed description of the different forms of polariscopes, the method of manipulation and the principles involved, the student is referred to any standard text-book of physics. A brief description follows: An ordinary ray of light vibrates in every direction. If such a ray is caused to pass through a "polarizing" Nicol prism it is resolved into *two rays*, one of which vibrates in every direction as before and a second ray which vibrates in *one plane only*. This latter ray is said to be *polarized*. Many organic substances (sugars, proteins, etc.) have the power of twisting or rotating this plane of polarized light, the extent to which the plane is rotated depending upon the number of molecules which the polarized light passes. Substances which possess this power are said to be "optically active." The *specific rotation* of a substance is the rotation expressed in degrees which is afforded by one gram of substance dissolved in 1 c.c. of water in a tube one decimeter in length. The specific rotation, $(\alpha)_D$, may be calculated by means of the following formula,

$$(\alpha)_D = \frac{\alpha}{p.l},$$

in which

D = sodium light.

α = observed rotation in degrees.

p = grams of substance dissolved in 1 c.c. of liquid.

l = length of the tube in decimeters.

If the specific rotation has been determined and it is desired to ascertain the per cent of the substance in solution, this may be obtained by the use of the following formula,

$$p = \frac{\alpha}{(\alpha)_D\, l}.$$

The value of p multiplied by 100 will be the percentage of the substance in solution.

An instrument by means of which the extent of the rotation may be determined is called a *polariscope* or *polarimeter*. Such an instrument designed especially for the examination of sugar solutions is termed a *saccharimeter* or *polarizing saccharimeter*. The form of polariscope in Fig. 3, above, consists essentially of a long barrel provided with a Nicol prism at either end (Fig. 4, p. 33). The solution under examination is contained in a tube which is placed between these two prisms. At the front end of the instrument is an adjusting eyepiece for focusing and a large recording disc which registers in degrees and fractions of a degree. The light is admitted into the far end of the instrument and is polarized by passing through a Nicol prism. This

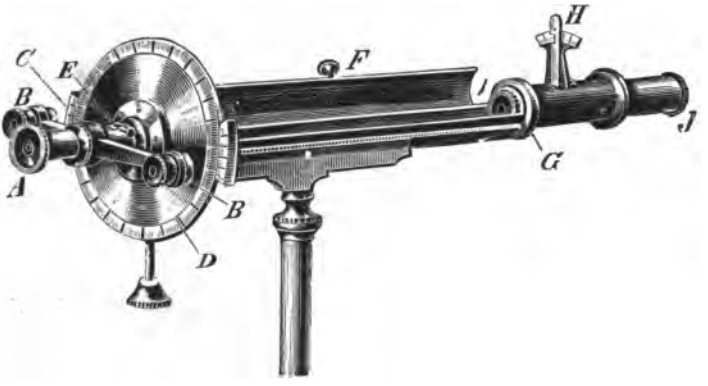


FIG. 3.—ONE FORM OF LAURENT POLARISCOPE.
B, Microscope for reading the scale; *C*, a vernier; *E*, position of the analyzing Nicol prism; *H*, polarizing Nicol prism in the tube below this point.

polarized ray then traverses the column of liquid within the tube mentioned above and if the substance is optically active the plane of the polarized ray is rotated to the right or left. Bodies rotating the

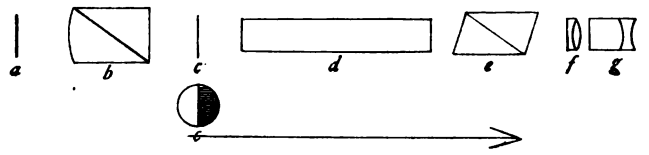


FIG. 4.—DIAGRAMMATIC REPRESENTATION OF THE COURSE OF THE LIGHT THROUGH THE LAURENT POLARISCOPE. (The direction is reversed from that of Fig. 3, p. 32.)
a, Bichromate plate to purify the light; *b*, the polarizing Nicol prism; *c*, a thin quartz plate covering one-half the field and essential in producing a second polarized plane; *d*, tube to contain the liquid under examination; *e*, the analyzing Nicol prism; *f* and *g*, ocular lenses.

ray to the right are called *dextro-rotatory* and those rotating it to the left *lævo-rotatory*.

Within the apparatus is a disc which is so arranged as to be without

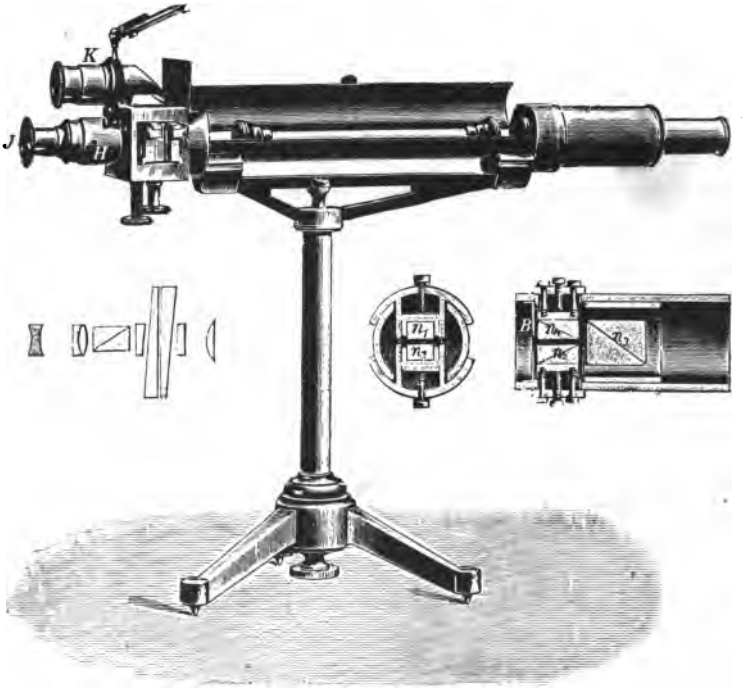


FIG. 5.—POLARISCOPE (SCHMIDT AND HAENSCH MODEL).

lines and uniformly light at zero. Upon placing the optically active substance in position, however, the plane of polarized light is rotated or turned and it is necessary to rotate the disc through a certain number

of degrees in order to secure the normal conditions, *i. e.*, "without lines and uniformly light." The difference between this reading and the *zero* is α or the observed rotation in degrees.

Polarizing saccharimeters are also constructed by which the percentage of sugar in solution is determined by making an observation and multiplying the value of each division on a horizontal sliding scale by the value of the division expressed in terms of dextrose. This factor may vary according to the instrument.

LÆVULOSE, $CH_2OH-(CHOH)_3-CO-CH_2OH$.

As already stated, lævulose, sometimes called fructose or fruit sugar, occurs widely disseminated throughout the plant kingdom in company with dextrose. Its reducing power is somewhat weaker than that of dextrose. Lævulose does not ordinarily occur in the urine in diabetes mellitus, but has been found in exceptional cases. With phenylhydrazine it forms the same osazone as dextrose. With methylphenylhydrazine, lævulose forms a characteristic methylphenyllævulosazone.

(For a further discussion of lævulose see the section on Hexoses, p. 21.)

Experiments on Lævulose.

1–13. Repeat these experiments as given under Dextrose, pages 22–30.

14. **Seliwanoff's Reaction.**—To 5 c.c. of Seliwanoff's reagent[1] in a test-tube add a few drops of a lævulose solution and heat the mixture to boiling. A positive reaction is indicated by the production of a red color and the separation of a red precipitate. The latter may be dissolved in alcohol to which it will impart a striking red color.

If the boiling be prolonged a similar reaction may be obtained with solutions of dextrose or maltose.

15. **Borchardt's Reaction.**—To about 5 c.c. of a solution of lævulose in a test-tube add an equal volume of 25 per cent hydro-

[1] Seliwanoff's reagent may be prepared by dissolving 0.05 gram of resorcin in 100 c.c. of dilute (1 : 2) hydrochloric acid.

chloric acid and a few crystals of resorcin. Heat to boiling and after the production of a red color, cool the tube under running water and transfer to an evaporating dish or beaker. Make the mixture slightly alkaline with *solid* potassium hydroxide, return it to a test-tube, add 2–3 c.c. of acetic ether and shake the tube vigorously. In the presence of lævulose, the acetic ether is colored yellow. (For further discussion of the test see Chapter XIX.)

16. **Formation of Methylphenyllævulosazone.**—To a solution of 1.8 grams of lævulose in 10 c.c. of water add 4 grams[1] of methylphenylhydrazine and enough alcohol to clarify the solution. Introduce 4 c.c of 50 per cent acetic acid and heat the mixture for 5–10 minutes on a boiling water-bath.[2] On standing 15 minutes at room temperature, crystallization begins and is complete in two hours. By scratching the sides of the flask or by inoculation, the solution quickly congeals to form a thick paste of reddish-yellow silky needles. These are the crystals of *methylphenyllævulosazone.* They may be recrystallized from hot 95 per cent alcohol and melt at 153° C.

GALACTOSE, $CH_2OH-(CHOH)_4-CHO$.

Galactose occurs with dextrose as one of the products of the hydrolysis of lactose. It is dextro-rotatory, forms an osazone with phenylhydrazine and ferments slowly with yeast. Upon oxidation with nitric acid galactose yields mucic acid, thus differentiating this monosaccharide from dextrose and lævulose. Lactose also yields mucic acid under these conditions. The mucic acid test may be used in urine examination to differentiate lactose and galactose from other reducing sugars.

Experiments on Galactose.

1. **Tollens' Reaction.**—To equal volumes of galactose solution and hydrochloric acid (sp. gr. 1.09) add a little phloroglucin, and heat the mixture on a boiling water-bath. Galactose, pentose and glycuronic acid will be indicated by the appearance of a red color. Galactose may be differentiated from the two latter substances in

[1] 3.66 grams if absolutely pure.
[2] Longer heating is to be avoided.

that its solutions exhibit no absorption bands upon spectroscopical examination.

2. **Mucic Acid Test.**—Treat 100 c.c. of the solution containing galactose with 20 c.c. of concentrated nitric acid (sp. gr. 1.4) and evaporate the mixture in a broad, shallow glass vessel on a boiling water-bath until the volume of the mixture has been reduced to about 20 c.c. At this point the fluid should be *clear*, and a fine white precipitate of *mucic acid* should form. If the percentage of galactose present is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will form. It is impossible to differentiate between galactose and lactose by this test, but the reaction serves to differentiate these two sugars from all other reducing sugars. Differentiate lactose from galactose by means of Barfoed's test (p. 30).

3. **Phenylhydrazine Reaction.**—Make the test according to directions given under Dextrose, 3 or 4, pages 23 and 24.

Pentoses, $C_5H_{10}O_5$.

In plants and more particularly in certain gums, very complex carbohydrates, called pentosans, occur. These pentosans through hydrolysis by acids may be transformed into pentoses. Pentoses do not ordinarily occur in the animal organism, but have been found in the urine of morphine habitués and others, their occurrence sometimes being a persistent condition without known cause. They are non-fermentable, have strong reducing power and form osazones with phenylhydrazine. Pentoses are an important constituent of the dietary of herbivorous animals. Glycogen is said to be formed after the ingestion of these sugars containing five oxygen atoms. This, however, has not been conclusively proven. On distillation with strong hydrochloric acid pentoses and pentosans yield furfurol, which can be detected by its characteristic red reaction with aniline-acetate paper.

ARABINOSE,

$$\begin{array}{l} CH_2OH \\ | \\ (CHOH)_3. \\ | \\ CHO \end{array}$$

Arabinose is one of the most important of the pentoses. The *l*-arabinose may be obtained from gum arabic, plum or cherry gum

by boiling for several hours, with 1–2 per cent sulphuric acid. This pentose is dextro-rotatory, forms an osazone and has reducing power. The *i*-arabinose has been isolated from the urine and yields an osazone which melts at 166°–168° C.

Experiments on Arabinose.

1. **Tollens' Reaction.**—To equal volumes of arabinose solution and hydrochloric acid (sp. gr. 1.09) add a little phloroglucin and heat the mixture on a boiling water-bath. Galactose, pentose or glycuronic acid will be indicated by the appearance of a red color. To differentiate between these bodies make a spectroscopic examination and look for the absorption band between *D* and *E* given by pentoses and glycuronic acid. Differentiate between the two latter bodies by the melting-points of their osazones.

Compare the reaction with that obtained with galactose (page 35).

2. **Orcin Test.**—Repeat 1, using orcin instead of phloroglucin. A succession of colors from red through reddish-blue to green is produced. A green precipitate is formed which is soluble in amyl alcohol and has absorption bands between *C* and *D*.

3. **Phenylhydrazine Reaction.**—Make this test on the arabinose solution according to directions given under Dextrose, 3 or 4, pages 23 and 24.

XYLOSE, $\begin{matrix} CH_2OH \\ | \\ (CHOH)_3 \\ | \\ CHO \end{matrix}$.

Xylose, or wood sugar, is obtained by boiling wood gums with dilute acids as explained under Arabinose, page 36. It is dextro-rotatory and forms an osazone.

Experiments on Xylose.

1–3. Same as for arabinose (see above).

RHAMNOSE, $C_6H_{12}O_5$.

Rhamnose or methyl-pentose is an example of a true carbohydrate which does not have the H and O atoms present in the proportion

to form water. Its formula is $C_6H_{12}O_6$. It has been found that rhamnose when ingested by rabbits or hens has a positive influence upon the formation of glycogen in those organisms.

DISACCHARIDES, $C_{12}H_{22}O_{11}$.

The disaccharides as a class may be divided into two rather distinct groups. The first group would include those disaccharides which are found in nature as such, *e. g.*, *sucrose* and *lactose* and the second group would include those disaccharides formed in the hydrolysis of more complex carbohydrates, *e. g.*, *maltose*, and *iso-maltose*.

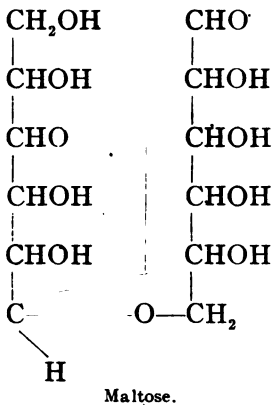
The disaccharides have the general formula $C_{12}H_{22}O_{11}$, to which, in the process of hydrolysis, a molecule of water is added causing the single disaccharide molecule to split into two monosaccharide (hexose) molecules. The products of the hydrolysis of the more common disaccharides are as follows:

Maltose = dextrose + dextrose.
Lactose = dextrose + galactose.
Sucrose = dextrose + lævulose.

All of the more common disaccharides *except sucrose* have the power of reducing certain metallic oxides in alkaline solution, notably those of copper and bismuth. This reducing power is due to the presence of the *aldehyde group* (—CHO) in the sugar molecule.

MALTOSE, $C_{12}H_{22}O_{11}$.

Maltose or malt sugar is formed in the hydrolysis of starch through the action of an enzyme, *vegetable amylase* (*diastase*), contained in sprouting barley or malt. Certain enzymes in the saliva and in the pancreatic juice may also cause a similar hydrolysis. Maltose is also an intermediate product of the action of dilute mineral acids upon starch. It is strongly dextro-rotatory, reduces metallic oxides in alkaline solution and is fermentable by yeast after being inverted (see Polysaccharides, page 42) by the enzyme *maltase* of the yeast. In common with the other disaccharides, maltose may be hydrolyzed with the formation of two molecules of monosaccharide. In this instance the products are two molecules of dextrose. With phenylhydrazine maltose forms an osazone, *maltosazone*. The following formula represents the probable structure of maltose:



Maltose.

Experiments on Maltose.

1–13. Repeat these experiments as given under Dextrose, pages 22–30.

ISO-MALTOSE, $C_{12}H_{22}O_{11}$.

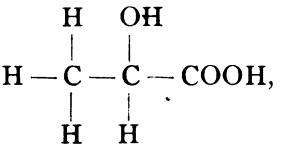
Iso-maltose, an isomeric form of maltose, is formed, along with maltose, by the action of diastase upon starch paste, and also by the action of hydrochloric acid upon dextrose. It also occurs with maltose as one of the products of salivary digestion. It is dextro-rotatory and with phenylhydrazine gives an osazone which is characteristic. Iso-maltose is very soluble and reduces the oxides of bismuth and copper in alkaline solution. Pure iso-maltose is probably only slightly fermentable.

LACTOSE, $C_{12}H_{22}O_{11}$.

Lactose or milk sugar occurs ordinarily only in milk, but has often been found in the urine of women during pregnancy and lactation. It may also occur in the urine of normal persons after the ingestion of unusually large amounts of lactose in the food. It has a strong reducing power, is dextro-rotatory and forms an osazone with phenylhydrazine. Upon hydrolysis lactose yields one molecule of dextrose and one molecule of galactose.

In the souring of milk the bacterium lactis and certain other

micro-organisms bring about lactic acid fermentation by transforming the lactose of the milk into lactic acid,



and alcohol. This same reaction may occur in the alimentary canal as the result of the action of putrefactive bacteria. In the preparation of kephyr and koumyss the lactose of the milk undergoes alcoholic fermentation, through the action of ferments other than yeast, and at the same time lactic acid is produced. Lactose and galatcose yield *mucic acid* on oxidation with nitric acid. This fact is made use of in urine analysis to facilitate the differentiation of these sugars from other reducing sugars.

Lactose is *not* fermentable by pure yeast.

Experiments on Lactose.

1–13. Repeat these experiments as given under Dextrose, pages 22–30.

14. **Mucic Acid Test.**—Treat 100 c.c. of the solution containing lactose with 20 c.c. of concentrated nitric acid (sp. gr. 1.4) and evaporate the mixture in a broad, shallow glass vessel on a boiling water-bath, until the volume of the mixture has been reduced to about 20 c.c. At this point the fluid should be *clear*, and a fine white precipitate of *mucic acid* should form. If the percentage of lactose present is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will appear. It is impossible to differentiate between lactose and galactose by this test, but the reaction serves to differentiate these two sugars from all other reducing sugars.

Differentiate lactose from galactose by means of Barfoed's test, page 30.

SUCROSE, $C_{12}H_{22}O_{11}$.

Sucrose, also called saccharose or cane sugar, is one of the most important of the sugars and occurs very extensively distributed in plants, particularly in the sugar cane, sugar beet, sugar millet and in certain palms and maples.

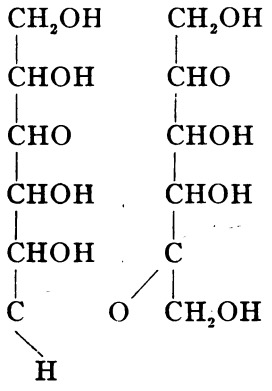
Sucrose is dextro-rotatory and upon hydrolysis, as before mentioned, the molecule of sucrose takes on a molecule of water and

breaks down into two molecules of monosaccharide. The monosaccharides formed in this instance are dextrose and lævulose. This is the reaction:

$$\underset{\text{Sucrose.}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Dextrose.}}{C_6H_{12}O_6} + \underset{\text{Lævulose.}}{C_6H_{12}O_6}.$$

This process is called *inversion* and may be produced by bacteria, enzymes, and certain weak acids. After this inversion the previously strongly dextro-rotatory solution becomes lævo-rotatory. This is due to the fact that the lævulose molecule is more strongly lævo-rotatory than the dextrose molecule is dextro-rotatory. The product of this inversion is called *invert sugar*.

Sucrose does *not* reduce metallic oxides in alkaline solution and forms *no* osazone with phenylhydrazine. It is not fermentable directly by yeast, but must first be *inverted* by the enzyme *sucrase* (*invertase* or *invertin*) contained in the yeast. The probable structure of sucrose may be represented by the following formula. Note the absence of any true sugar group or free ketone or aldehyde group.



Sucrose.

Experiments on Sucrose.

1–13. Repeat these experiments according to the directions given under Dextrose, pages 22–30.

14. **Inversion of Sucrose.**—To 25 c.c. of sucrose solution in a beaker add 5 drops of concentrated HCl and boil one minute. Cool the solution, render alkaline with *solid* KOH and upon the resulting fluid repeat experiments 3 (or 4) and 11 as given under Dextrose, pages 23–25. Explain the results.

15. **Production of Alcohol by Fermentation.**—Prepare a strong (10–20 per cent) solution of sucrose, add a small amount of egg albumin or commercial peptone and introduce the mixture into a bottle of appropriate size. Add yeast, and by means of a bent tube inserted through a stopper into the neck of the bottle, conduct the escaping gas into water. As fermentation proceeds readily in a warm place the escaping gas may be collected in a eudiometer tube and examined. When the activity of the yeast has practically ceased, filter the contents of the bottle into a flask and distil the mixture. Catch the first portion of the distillate separately and test for alcohol by one of the following reactions:

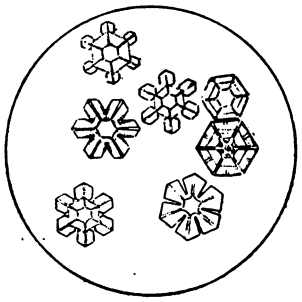


FIG. 6.—IODOFORM. (*Autenrieth.*)

(*a*) *Iodoform Test.*—Render 2–3 c.c. of the distillate alkaline with potassium hydroxide solution and add a few drops of iodine solution. Heat gently and note the formation of iodoform crystals. Examine these crystals under the microscope and compare them with those in Fig. 6.

(*b*) *Aldehyde Test.*—Place 5 c.c. of the distillate in a test-tube, add a few drops of potassium dichromate solution, $K_2Cr_2O_7$, and render it acid with dilute sulphuric acid. Boil the acid solution and note the odor of aldehyde.

TRISACCHARIDES, $C_{18}H_{32}O_{16}$.

RAFFINOSE.

This trisaccharide, also called melitose, or melitriose occurs in cotton seed, Australian manna, and in the molasses from the preparation of beet sugar. It is dextro-rotatory, does not reduce Fehling's solution, and is only partially fermentable by yeast.

Raffinose may be hydrolyzed by weak acids the same as the polysaccharides are hydrolyzed, the products being lævulose and melibiose; further hydrolysis of the melibiose yields dextrose and galactose.

POLYSACCHARIDES, $(C_6H_{10}O_5)_x$.

In general the polysaccharides are amorphous bodies, a few, however, are crystallizable. Through the action of certain enzymes or weak acids the polysaccharides may be hydrolyzed with the formation of monosaccharides. As a class the polysaccharides are quite insolu-

ble and are non-fermentable until inverted. By inversion is meant the hydrolysis of disaccharide or polysaccharide sugars to form monosaccharides, as indicated in the following equations:

(*a*) $C_{12}H_{22}O_{11}+H_2O=2(C_6H_{12}O_6)$.

(*b*) $C_6H_{10}O_5+H_2O=C_6H_{12}O_6$.

STARCH, $(C_6H_{10}O_5)_x$.

Starch is widely distributed throughout the vegetable kingdom, occurring in grains, fruits, and tubers. It occurs in granular form, the microscopical appearance being typical for each individual starch. The granules, which differ in size according to the source, are composed of alternating concentric rings of granulose and cellulose. Ordinary starch is insoluble in cold water, but if boiled with water the cell walls are ruptured and *starch paste* results. In general starch gives a *blue* color with iodine.

Starch is acted upon by amylases, *e. g.*, salivary amylase (*ptyalin*) and pancreatic amylase (*amylopsin*), with the formation of *soluble starch, erythro-dextrin, achroo-dextrins, maltose, iso-maltose* and *dextrose* (see Salivary Digestion, page 53). Maltose is the principal end-product of this enzyme action. Upon boiling a starch solution with a dilute mineral acid a series of similar bodies is formed, but under these conditions *dextrose* is the principal end-product.

Experiments on Starch.

1. **Preparation of Potato Starch.**—Pare a raw potato, comminute it upon a fine grater, mix with water, and "whip up" the pulped material vigorously before straining it through cheese cloth or gauze to remove the coarse particles. The starch rapidly settles to the bottom and can be washed by repeated decantation. Allow the compact mass of starch to drain thoroughly and spread it out on a watch glass to dry in the air. If so desired this preparation may be used in the experiments which follow.

2. **Microscopical Examination.**—Examine microscopically the granules of the various starches submitted and compare them with those shown in Figs. 7–17, page 44. The suspension of the granules in a drop of water will facilitate the microscopical examination.

3. **Solubility.**—Try the solubility of one form of starch in each of the ordinary solvents (see page 22). If uncertain regarding the

FIG. 7.—POTATO.

FIG. 8.—BEAN.

FIG. 9.—ARROWROOT.

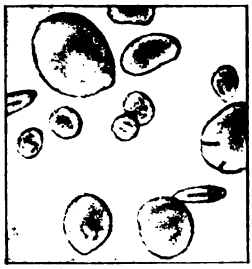


FIG. 10.—RYE.

FIG. 11.—BARLEY.

FIG. 12.—OAT.

FIG. 13.—BUCKWHEAT.

FIG. 14.—MAIZE.

FIG. 15.—RICE.

FIG. 16.—PEA.

FIG. 17.—WHEAT.

STARCH GRANULES FROM VARIOUS SOURCES. (*Leffman and Beam.*)

solubility in any reagent, filter and test the filtrate with iodine solution as given under 5 below. The production of a blue color would indicate that the starch had been dissolved by the solvent.

4. **Iodine Test.**—Place a few granules of starch in one of the depressions of a porcelain test-tablet and treat with a drop of a dilute solution of iodine in potassium iodide. The granules are colored blue due to the formation of so-called *iodide of starch.* The cellulose of the granule is not stained as may be seen by examining microscopically.

5. **Iodine Test on Starch Paste.**[1]—Repeat the iodine test using the starch paste. Place 2–3 c.c. of starch paste[2] in a test-tube, add a drop of the dilute iodine solution and observe the production of a blue color. Heat the tube and note the disappearance of the color. It reappears on cooling.

In similar tests note the influence of alcohol and of alkali upon the so-called iodide of starch.

The composition of the iodide of starch is not definitely known.

6. **Fehling's Test.**—On starch paste (see page 27).

7. **Hydrolysis of Starch.**—Place about 25 c.c. of starch paste in a small beaker, add 10 drops of concentrated HCl, and boil. By means of a small pipette, at the end of each minute, remove a drop of the solution to the test-tablet and make the regular iodine test. As the testing proceeds the blue color should gradually fade and finally disappear. . At this point, after cooling and neutralizing with solid KOH, Fehling's test (see page 27) should give a positive result due to the formation of a reducing sugar from the starch. Make the phenylhydrazine test upon some of the hydrolyzed starch. What sugar has been formed?

8. **Influence of Tannic Acid.**—Add an excess of tannic acid solution to a small amount of starch paste in a test-tube. The liquid will become strongly opaque and ordinarily a yellowish-white precipitate is produced. Compare this result with the result of the similar experiment on dextrin (page 48).

9. **Diffusibility of Starch Paste.**—Test the diffusibility of starch paste through animal membrane or parchment paper, making a dialyzer like one of the models shown in Fig. 1, page 25.

[1] *Preparation of Starch Paste.*—Grind 2 grams of starch powder in a mortar with a small amount of cold water. Bring 200 c.c. of water to the boiling-point and add the starch mixture from the mortar with continuous stirring. Bring again to the boiling-point and allow it to cool. This makes an approximate 1 per cent starch paste which is a very satisfactory strength for general use.

[2] For this particular test a starch paste of very satisfactory strength may be made by mixing 1 c.c. of a 1 per cent starch paste with 100 c.c. of water.

INULIN, $(C_6H_{10}O_5)_x$.

Inulin is a polysaccharide which may be obtained as a white, odorless, tasteless powder from the tubers of the artichoke, elecampane, or dahlia. It has also been prepared from the roots of chicory, dandelion, and burdock. It is very slightly soluble in cold water and quite easily soluble in hot water. In cold alcohol of 60 per cent or over it is practically insoluble. Inulin gives a negative reaction with iodine solution. The "yellow" color reaction with iodine mentioned in many books is doubtless merely the normal color of the iodine solution. It is very difficult to prepare inulin which does not reduce Fehling's solution slightly. This reducing power may be due to an impurity. Practically all commercial preparations of inulin possess considerable reducing power.

Inulin is lævo-rotatory and upon hydrolysis by acids or by the enzyme *inulase* it yields the monosaccharide lævulose which readily reduces Fehling's solution. The ordinary amylolytic enzymes occurring in the animal body do not digest inulin.

Experiments on Inulin.

1. **Solubility.**—Try the solubility of inulin powder in each of the ordinary solvents. If uncertain regarding the solubility in any reagent, filter and neutralize the filtrate if it is alkaline in reaction. Add a drop of concentrated hydrochloric acid to the filtrate and boil it for one minute. Render the solution neutral or slightly alkaline with *solid* potassium hydroxide and try Fehling's test. What is the significance of a positive Fehling's test in this connection?

2. **Iodine Test.**—(*a*) Place 2–3 c.c. of the inulin solution in a test-tube and add a drop of dilute iodine solution. What do you observe?

(*b*) Place a small amount of inulin powder in one of the depressions of a test-tablet and add a drop of dilute iodine solution. Is the effect any different from that observed above?

3. **Molisch's Reaction.**—Repeat this test according to directions given under Dextrose, 2, page 22.

4. **Fehling's Test.**—Make this test on the inulin solution according to the instructions given under Dextrose, page 27. Is there any reduction?[1]

5. **Hydrolysis of Inulin.**—Place 5 c.c. of inulin solution in a test-tube, add a drop of concentrated hydrochloric acid and boil it for one minute. Now cool the solution, neutralize it with concentrated

[1] See the discussion of the properties of inulin, above.

KOH and test the reducing action of 1 c.c. of the solution upon 1 c.c. of diluted (1 :4) Fehling's solution. Explain the result.[1]

GLYCOGEN, $(C_6H_{10}O_5)_x$.

(For discussion and experiments see Muscular Tissue, Chapter XV.)

LICHENIN, $(C_6H_{10}O_5)_x$.

Lichenin may be obtained from *Cetraria islandica* (Iceland moss). It forms a difficultly soluble jelly in cold water and an opalescent solution in hot water. It is optically inactive and gives no color with iodine. Upon hydrolysis with dilute mineral acids lichenin yields dextrins and dextrose. It is said to be most nearly related chemically to starch. Saliva, pancreatic juice, malt diastase and gastric juice have no noticeable action on lichenin.

DEXTRIN, $(C_6H_{10}O_5)_x$.

The dextrins are the bodies formed midway in the stages of the hydrolysis of starch by weak acids or an enzyme. They are amorphous bodies which are easily soluble in water, acids, and alkalis, but are insoluble in alcohol or ether. Dextrins are dextro-rotatory and are not fermentable by yeast.

The dextrins may be hydrolyzed by dilute acids to form dextrose. With iodine one form of dextrin (erythro-dextrin) gives a red color. Their power to reduce Fehling's solution is questioned.

Experiments on Dextrin.

1. **Solubility.**—Test the solubility of pulverized dextrin in the ordinary solvents (see page 22).

2. **Iodine Test.**—Place a drop of dextrin solution in one of the depressions of the test-tablet and add a drop of a dilute solution of iodine in potassium iodide. A red color results due to the formation of the *red iodide of dextrin.* If the reaction is not sufficiently pronounced make a stronger solution from pulverized dextrin and repeat the test. The solution should be slightly acid to secure the best results.

Make proper tests to show that the *red iodide of dextrin* is influenced by heat, alkali, and alcohol in a similar manner to the *blue iodide of starch* (see page 45).

[1] If the inulin solution gave a positive Fehling test in the last experiment it will be necessary to check the hydrolysis experiment as follows: To 5 c.c. of the inulin solution in a test-tube add one drop of concentrated hydrochloric acid, neutralize with concentrated KOH solution and test the reducing action of 1 c.c. of the resulting solution upon 1 c.c. of diluted (1 : 4) Fehling's solution. This will show the normal reducing power of the inulin solution. In case the inulin was hydrolyzed, the Fehling's test in the hydrolysis experiment should show a more pronounced reduction than that observed in the check experiment.

3. **Fehling's Test.**—See if the dextrin solution will reduce Fehling's solution.

4. **Hydrolysis of Dextrin.**—Take 25 c.c. of dextrin solution in a small beaker, add 5 drops of dilute hydrochloric acid, and boil. By means of a small pipette, at the end of each minute, remove a drop of the solution to one of the depressions of the test-tablet and make the iodine test. The power of the solution to produce a color with iodine should rapidly disappear. When a negative reaction is obtained cool the solution and neutralize it with concentrated potassium hydroxide. Try Fehling's test (see page 27). This reaction is now strongly positive, due to the formation of a reducing sugar. Determine the nature of the sugar by means of the phenylhydrazine test (see pages 23 and 24).

5. **Influence of Tannic Acid.**—Add an excess of tannic acid solution to a small amount of dextrin solution in a test-tube. No precipitate forms. This result differs from the result of the similar experiment upon starch (see Starch, 8, page 45).

6. **Diffusibility of Dextrin.**—(See Starch, 9, page 45.)

7. **Precipitation by Alcohol.**—To about 50 c.c. of 95 per cent alcohol in a small beaker add about 10 c.c. of a *concentrated* dextrin solution. Dextrin is thrown out of solution as a gummy white precipitate. Compare the result with that obtained under Dextrose, 5, page 45.

CELLULOSE, $(C_6H_{10}O_5)_x$.

This complex polysaccharide forms a large portion of the cell wall of plants. It is extremely insoluble and its molecule is much more complex than the starch molecule. The best quality of filter paper and the ordinary absorbent cotton are good types of cellulose.

Experiments on Cellulose.

1. **Solubility.**—Test the solubility of cellulose in the ordinary solvents (see page 22).

2. **Iodine Test.**—Add a drop of dilute iodine solution to a few shreds of cotton on a test-tablet. Cellulose differs from starch and dextrin in giving *no color* with iodine.

3. **Formation of Amyloid.**[1]—Add 10 c.c. of dilute and 5 c.c. of concentrated H_2SO_4 to some absorbent cotton in a test-tube. When entirely dissolved (without heating) pour one-half of the solution into another test-tube, cool it and dilute with water. Amyloid forms as a gummy precipitate and gives a brown or blue coloration with iodine.

[1] This body derives its name from *amylum* (starch) and is not to be confounded with amyloid, the glycoprotein.

After allowing the second portion of the acid solution of cotton to stand about 10 minutes, dilute it with water in a small beaker and boil for 15–30 minutes. Now cool, neutralize with *solid* KOH and test with Fehling's solution. Dextrose has been formed from the cellulose by the action of the acid.

4. **Schweitzer's Solubility Test.**—Place a little absorbent cotton in a test-tube, add Schweitzer's reagent,[1] and stir the cellulose with a glass rod. When completely dissolved acidify the solution with acetic acid. An amorphous precipitate of cellulose is produced.

5. **Cross and Bevan's Solubility Test.**[2]—Place a little absorbent cotton in a test-tube, add Cross and Bevan's reagent,[3] and stir the cellulose with a glass rod. When solution is complete reprecipitate the celluose with 95 per cent alcohol.

REVIEW OF CARBOHYDRATES.

In order to facilitate the student's review of the carbohydrates, the preparation of a chart similar to the appended model is recommended. The signs + and − may be conveniently used to indicate positive and negative reaction. Only those carbohydrates which

MODEL CHART FOR REVIEW PURPOSES.

Carbohydrate.	Solubility.	Iodine Test.	Moore's Test.	Trommer's Test.	Fehling's Test.	Boettger's Test.	Nylander's Test.	Barfoed's Test.	Seliwanoff's Reaction.	Molisch's Reaction.	Mucic Acid Test.	Borchardt's Reaction.	Precipitation by Alcohol.	Osazone.	Rotation.	Diffusibility.	Fermentation.	Remarks.
Dextrose.		−	+							+						+	+	
Lævulose.		−								+		+					+	
Maltose.		−			−	+	+	+									+	
Iso-maltose.																		
Lactose.		−								+							−	
Sucrose.		−								+							+	
Starch.		+														−	−	
Inulin.		?															−	
Glycogen.																	−	
Dextrin.																	−	
Cellulose.																	−	

[1] Schweitzer's reagent is made by adding potassium hydroxide to a solution of cupric sulphate which contains some ammonium chloride. A precipitate of cupric hydroxide forms and this is filtered off, washed, and 3 grams of the moist cupric hydroxide brought into solution in a liter of 20 per cent ammonium hydroxide.

[2] Cross and Bevan: *Chemical News*, 63, p. 66.

[3] Cross and Bevan's reagent may be prepared by combining two parts of concentrated hydrochloric acid and one part of zinc chloride, *by weight*.

SCHEME FOR THE DETECTION OF CARBOHYDRATES.

If the solution is alkaline, neutralize or make faintly acid with HCl. Test a few drops on a test-tablet, or in a test-tube with iodine solution.

- Blue color indicates *starch*.
- Reddish color indicates
  - *Erythro-dextrin* if "unknown" solution was transparent.
  - *Glycogen* if "unknown" solution was opalescent.
- No color with iodine solution indicates *sugar*.
  - Test the "unknown" solution by Fehling's test.
    - Reduction indicates *dextrose*, *maltose*, *lactose*, *lævulose* or *galactose*.
      - Test by Barfoed's reagent.
        - Reduction indicates *dextrose*, *lævulose* or *galactose*.
          - Test by Seliwanoff's reaction or Borchardt's reaction. Positive test indicates *lævulose;* negative test indicates *dextrose* or *galactose*.
            - Differentiate between *dextrose* and *galactose* by the mucic acid test, p. 36, Tollens' reaction, p. 35, or the phenylhydrazine reaction, p. 23.
        - No reduction indicates *lactose* or *maltose*.
          - Differentiate between *lactose* and *maltose* by the mucic acid test, p. 36, the phenylhydrazine reaction or the fermentation test.
    - No reduction indicates the possible presence of *sucrose*.
      - To 5 c.c. of the "unknown" solution in a test-tube add one drop of concentrated hydrochloric acid and heat to boiling for one minute. Cool, neutralize with potassium hydroxide solution and test by Fehling's test.
        - Reduction indicates that dextrose has been formed from the sucrose of the "unknown" solution.
        - No reduction indicates the absence of sucrose in the "unknown" solution.

If uncertain as to the differentiatio between dextrin and glycogen add basic lead acetate to some of the "unknown" solution.

- No precipitate i dicates *erythro-dextrin*.
- Precipitate indicates *glycogen*.

are of greatest importance from the standpoint of physiological chemistry have been included in the chart.

"Unknown" Solutions of Carbohydrates.

At this point the student will be given several "unknown" solutions, each solution containing one or more of the carbohydrates studied. He will be required to detect, by means of the tests on the preceding pages, each carbohydrate constituent of the several "unknown" solutions and hand in, to the instructor, a written report of his findings, on slips furnished by the laboratory.

The scheme given on page 50 may be of use in this connection.

CHAPTER III.

SALIVARY DIGESTION.

THE saliva is secreted by three pairs of glands, the submaxillary, sublingual, and parotid, reinforced by numerous small glands called buccal glands. The saliva secreted by each pair of glands possesses certain definite characteristics peculiar to itself. For instance, in man the parotid glands ordinarily secrete a thin, watery fluid, the submaxillary glands secrete a somewhat thicker fluid containing mucin, while the product of the sublingual glands has a more mucilaginous character. The saliva as collected from the mouth is the combined product of all the glands mentioned.

The saliva may be induced to flow by many forms of stimuli, such as *chemical*, *mechanical*, *electrical*, *thermal*, and *psychical*, the nature and amount of the secretion depending, to a limited degree, upon the particular class of stimuli employed as well as upon the character of the individual stimulus. For example, in experiments upon dogs it has been found that the mechanical stimulus afforded by dropping several pebbles into the animal's mouth caused the flow of but one or two drops of saliva, whereas the mechanical stimulus afforded by sand thrown into the mouth induced a copius flow of a thin watery fluid. Again, when ice-water or snow was placed in the animal's mouth no saliva was seen, while an acid or anything possessing a bitter taste, which the dog wished to reject, caused a free flow of the thin saliva. On the other hand, when articles of food were placed in the dog's mouth the animal secreted a thicker saliva having a higher mucin content—a fluid which would lubricate the food and assist in the passage of the bolus through the œsophagus. It was further found that by simply drawing the attention of the animal to any of the substances named above, results were obtained similar to those secured when the substances were actually placed in the animal's mouth. For example, when a pretense was made of throwing sand into the dog's mouth, a watery saliva was secreted, whereas food under the same conditions excited a thicker and more slimy secretion. The exhibition of dry food, in which the dog had no particular interest (dry bread) caused the secretion of a large amount of watery saliva, while the presentation of moist food, which was eagerly desired by the

animal, called forth a much smaller secretion, slimy in character. These experiments show it to be rather difficult to differentiate between the influence of physiological and psychical stimuli.

The amount of saliva secreted by an adult in 24 hours has been variously placed, as the result of experiment and observation, between 1000 and 1500 c.c., the exact amount depending, among other conditions, upon the character of the food.

The saliva ordinarily has a weak, alkaline reaction to litmus, but becomes acid, in some instances, 2–3 hours after a meal or during fasting. The alkalinity is due principally to di-sodium hydrogen phosphate (Na_2HPO_4) and its average alkalinity may be said to be equivalent to 0.08–0.1 per cent sodium carbonate. The saliva is the most dilute of all the digestive secretions, having an average specific gravity of 1.005 and containing only 0.5 per cent of solid matter. Among the solids are found albumin, globulin, mucin, urea, the enzymes salivary amylase (ptyalin) and maltase, phosphates, and other inorganic constitutents. Potassium thiocyanate, KSCN, is also generally present in the saliva. It has been claimed that this substance is present in greatest amount in the saliva of habitual smokers. The significance of thiocyanate in the saliva is not known; it probably comes from the ingested thiocyanates and from the breaking down of protein material.

The so-called tartar formation on the teeth is composed almost entirely of calcium phosphate with some calcium carbonate, mucin, epithelial cells, and organic débris derived from the food. The calcium salts are held in solution as acid salts, and are probably precipitated by the ammonia of the breath. The various organic substances just mentioned are carried down in the precipitation of the calcium salts.

The principal enzyme of the saliva is known as *salivary amylase* or *ptyalin*. This is an *amylolytic* enzyme (see p. 3), so called because it possesses the property of transforming complex carbohydrates such as starch and dextrin into simpler bodies. The action of salivary amylase is one of hydrolysis and through this action a series of simpler bodies are formed from the complex starch. The first product of the action of the ptyalin of the saliva upon starch paste is *soluble starch* (amidulin) and its formation is indicated by the disappearance of the opalescence of the starch solution. This body resembles true starch in giving a blue color with iodine. Next follows the formation, in succession, of a series of dextrins, called *erythro-dextrin*, *α-achroo-dextrin*, *β-achroo-dextrin*, and *γ-achroo-dextrin*, the *erythro-dextrin*

being formed directly from *soluble starch* and later being itself transformed into *α-achroo-dextrin* from which in turn are produced *β-achroo-dextrin* and *γ-achroo-dextrin*. Accompanying each dextrin a small amount of iso-maltose is formed, the quantity of iso-maltose growing gradually larger as the process of transformation progresses. (Erythro-dextrin gives a red color with iodine, the other dextrins give no color.) The next stage is the transformation of the *γ-achroo-dextrin* into *iso-maltose* and subsequently the transformation of the iso-maltose into maltose, the latter being the principal end-product of the salivary digestion of starch. At this point a small amount of *dextrose* is formed from the maltose, through the action of the enzyme *maltase*.

Salivary amylase acts in alkaline, neutral, or combined acid solutions. It will act in the presence of relatively strong *combined* HCl (see page 409), whereas a trace (0.003 per cent to 0.006 per cent) of ordinary *free* hydrochloric acid will not only prevent the action but will destroy the enzyme. By sufficiently increasing the alkalinity of the saliva to litmus, the action of the salivary amylase is inhibited. It has recently been shown by Cannon that salivary digestion may proceed for a considerable period after the food reaches the stomach, owing to the slowness with which the contents are thoroughly mixed with the acid gastric juice and the consequent tardy destruction of the enzyme. Food in the pyloric end of the stomach is soon mixed with the gastric secretion, but food in the cardiac end is not mixed with the acid gastric juice for a considerable period of time, and in this region during that time salivary digestion may proceed undisturbed.

Maltase, sometimes called glucase, is the second enzyme of the saliva. It is an amylolytic enzyme whose principal function is the splitting of maltose into dextrose. Besides occurring in the saliva it is also present in the pancreatic and intestinal juices. For experimental purposes the enzyme is ordinarily prepared from corn. The principles of the "reversibility" of enzyme action were first demonstrated in connection with maltase by Croft Hill.

Microscopical examination of the saliva reveals salivary corpuscles, bacteria, food débris, epithelial cells, mucus, and fungi. In certain pathological conditions of the mouth, pus cells, and blood corpuscles may be found in the saliva.

Experiments on Saliva.

A satisfactory method of obtaining the saliva necessary for the experiments which follow is to chew a small piece of pure paraffin wax, thus stimulating the flow of the secretion, which may be collected

in a small beaker. Filtered saliva is to be used in every experiment except for the microscopical examination.

1. **Microscopical Examination.**—Examine a drop of unfiltered saliva microscopically and compare with Fig. 18 below.

2. **Reaction.**—Test the reaction to litmus. Alkaline

3. **Specific Gravity.**—Partially fill a urinometer cylinder with saliva, introduce the urinometer, and observe the reading.

4. **Test for Mucin.**—To a small amount of saliva in a test-tube add 1–2 drops of dilute acetic acid. Mucin is precipitated.

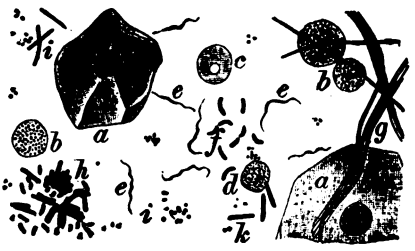


FIG. 18.—MICROSCOPICAL CONSTITUENTS OF SALIVA.
a, Epithelial cells; *b*, salivary corpuscles; *c*, fat drops; *d*, leucocytes; *e*, *f* and *g*, bacteria; *h*, *i* and *k*, fission-fungi.

5. **Biuret Test.**[1]—Render a little saliva alkaline with an equal volume of KOH and add a few drops of a *very dilute* (2–5 drops in a test-tube of water) cupric sulphate solution. The formation of a purplish-violet color is due to mucin.

6. **Millon's Reaction.**[2]—Add a few drops of Millon's reagent to a little saliva. A light yellow precipitate formed by the mucin gradually turns red upon being *gently* heated.

7. **Preparation of Mucin.**—Pour 25 c.c. of saliva into 100 c.c. of 95 per cent alcohol, stirring constantly. Cover the vessel and allow the precipitate to stand at least 12 hours. Pour off the supernatant liquid, collect the precipitate on a filter and wash it, in turn, with alcohol and ether. Finally dry the precipitate, remove it from the paper and make the following tests on the mucin: (*a*) Test its solubility in the ordinary solvents (see page 22); (*b*) Millon's reaction; (*c*) dissolve a small amount in KOH, and try the biuret test on the solution; (*d*) boil the remainder, with 10–25 c.c. of water to which 5 c.c. of dilute HCl has been added, until the solution becomes brownish. Cool, render alkaline with *solid* KOH, and test by Fehling's solution. A reduction should take place. Mucin is what is known as a conju-

[1] The significance of this reaction is pointed out on page 90.
[2] The significance of this reaction is pointed out on page 88.

gated protein or glycoprotein (see p. 85) and upon boiling with the acid the carbohydrate group in the molecule has been split off from the protein portion and its presence is indicated by the reduction of Fehling's solution.

8. **Inorganic Matter.**—Test for chlorides, phosphates, sulphates, and calcium. For chlorides, acidify with HNO_3 and add $AgNO_3$. For phosphates, acidify with HNO_3, heat and add molybdic solution.[1] For sulphates, acidify with HCl and add $BaCl_2$ and warm. For calcium, acidify with acetic acid, CH_3COOH, and add ammonium oxalate, $(NH_4)_2C_2O_4$.

9. **Viscosity Test.**—Place filter papers in two funnels, and to each add an equal quantity of starch paste (5 c.c.). Add a few drops of saliva to one lot of paste and an equivalent amount of water to the other. Note the progress of filtration in each case. Why does one solution filter more rapidly than the other?

10. **Test for Nitrites.**—Add 1–2 drops of dilute H_2SO_4 to a little saliva and thoroughly stir. Now add a few drops of a potassium iodide solution and some starch paste. Nitrous acid is formed which liberates iodine, causing the formation of the blue iodide of starch.

11. **Thiocyanate Tests.**—(*a*) *Ferric Chloride Test.*—To a little saliva in a small porcelain crucible, or dish, add a few drops of dilute ferric chloride and acidify slightly with HCl. Red ferric thiocyanate forms. To show that the red coloration is not due to iron phosphate add a drop of $HgCl_2$ when *colorless* mercuric thiocyanate forms.

(*b*) *Solera's Reaction.*—This test depends upon the liberation of iodine through the action of thiocyanate upon iodic acid. Moisten a strip of *starch paste-iodic acid* test paper[2] with a little saliva. If thiocyanate be present the test paper will assume a blue color, due to the liberation of iodine and the subsequent formation of the so-called iodide of starch.

12. **Digestion of Starch Paste.**—To 25 c.c. of starch paste in a small beaker, add 5 drops of saliva and stir thoroughly. At intervals of a minute remove a drop of the solution to one of the depressions in a test-tablet and test by the iodine test. If the blue color with iodine still forms after 5 minutes, add another 5 drops of saliva. The opal-

[1] Molybdic solution is prepared as follows, the parts being by weight:
1 part molybdic acid.
4 parts ammonium hydroxide (sp. gr. 0.96).
15 parts nitric acid (sp. gr. 1.2).

[2] This test paper is prepared as follows: Saturate a good quality of filter paper with 0.5 per cent starch paste to which has been added sufficient iodic acid to make a 1 per cent solution of iodic acid and allow the paper to dry in the air. Cut it in strips of suitable size and preserve for use.

escence of the starch solution should soon disappear, indicating the formation of *soluble starch* which gives a blue color with iodine. This body should soon be transformed into *erythro-dextrin* which gives a red color with iodine, and this in turn should pass into *achroo-dextrin* which gives no color with iodine. This is called the *achromic point.* When this point is reached test by Fehling's test to show the production of a reducing body. A positive Fehling's test may be obtained while the solution still reacts red with iodine inasmuch as some *iso-maltose* is formed from the soluble starch coincidently with the formation of the *erythro-dextrin.* How long did it take for a complete transformation of the starch?

13. **Digestion of Dry Starch.**—In a test-tube shake up a small amount of *dry starch* with a little water. Add a few drops of saliva, mix well, and allow to stand. After 10–20 minutes filter and test the filtrate by Fehling's test. What is the result and why?

14. **Digestion of Inulin.**—To 5 c.c. of inulin solution in a test-tube add 10 drops of saliva and place the tube in the water-bath at 40° C. After one-half hour test the solution by Fehling's test.[1] Is any reducing substance present? What do you conclude regarding the salivary digestion of inulin?

15. **Influence of Temperature.**—In each of four tubes place about 5 c.c. of starch paste. Immerse one tube in cold water from the faucet, keep a second at room temperature, and place a third on the water-bath at 40° C. Now add to the contents of each of these three tubes two drops of saliva and shake well; to the contents of the fourth tube add two drops of *boiled* saliva. Test frequently by the iodine test, using the test-tablet, and note in which tube the most rapid digestion occurs. Explain the results.

16. **Influence of Dilution.**—Take a series of six test-tubes each containing 9 c.c. of water. Add 1 c.c. of saliva to tube 1 and shake thoroughly. Remove 1 c.c. of the solution from tube 1 to tube 2 and after mixing thoroughly remove 1 c.c. from tube 2 to tube 3. Continue in this manner until you have 6 saliva solutions of gradually decreasing strength. Now add starch paste in equal amounts to each tube, mix very thoroughly, and place on the water-bath at 40° C. After 10–20 minutes test by both the iodine and Fehling's tests. In how great dilution does your saliva act?

17. **Influence of Acids and Alkalis.**—(*a*) *Influence of Free Acid.*—Prepare a series of six tubes in each of which is placed 4 c.c.

[1] If the inulin solution gives a reduction before being acted upon by the saliva it will be necessary to determine the extent of the original reduction by means of a "check" test (see page 47).

of one of the following strengths of *free* HCl: 0.2 per cent, 0.1 per cent, 0.05 per cent, 0.025 per cent, 0.0125 per cent and 0.006 per cent. Now add 2 c.c. of starch paste to each tube and shake them thoroughly. Complete the solutions by adding 2 c.c. of saliva to each and repeat the shaking. The *total acidity* of this series would be as follows: 0.1 per cent, 0.05 per cent, 0.025 per cent, 0.0125 per cent, 0.006 per cent and 0.003 per cent. Place these tubes on the water-bath at 40° C. for 10–20 minutes. Divide the contents of each tube into two parts, testing one part by the iodine test and testing the other, after neutralization, by Fehling's test. What do you find?

(*b*) *Influence of Combined Acid.*—Repeat the first three experiments of the above series using *combined* hydrochloric acid (see page 409) instead of the *free* acid. How does the action of the *combined acid* differ from that of the *free acid?*

(*c*) *Influence of Alkali.*—Repeat the first four experiments under (*a*) replacing the HCl by 2 per cent, 1 per cent, 0.5 per cent and 0.25 per cent Na_2CO_3. Neutralize the alkalinity before trying the iodine test (see Starch, 5, page 45).

(*d*) *Nature of the Action of Acid and Alkali.*—Place 2 c.c. of saliva and 2 c.c. of 0.2 per cent HCl in a test-tube and leave for 15 minutes. Neutralize the solution, add 4 c.c. of starch paste and place the tube on the water-bath at 40° C. In 10 minutes test by the iodine and Fehling's tests and explain the result. Repeat the experiment, replacing the 0.2 per cent HCl by 2 per cent Na_2CO_3. What do you deduce from these two experiments?

18. **Influence of Metallic Salts, etc.**—In each of a series of tubes place 4 c.c. of starch paste and 1/2 c.c. of one of the solutions named below. Shake well, add 1/2 c.c. of saliva to each tube, thoroughly mix, and place on the water-bath at 40° C. for 10–20 minutes. Show the progress of digestion by means of the iodine and Fehling tests. Use the following chemicals: (1) *Metallic salts*, 10 per cent plumbic acetate, 2 per cent cupric sulphate, 5 per cent ferric chloride, 8 per cent mercuric chloride; (2) *Neutral salts*, 10 per cent sodium chloride, 10 per cent magnesium sulphate, 3 per cent barium chloride, 10 per cent Rochelle salt. Also try the influence of 2 per cent carbolic acid, 95 per cent alcohol, and ether and chloroform. What are your conclusions?

19. **Excretion of Potassium Iodide.**—Ingest a small dose of potassium iodide (0.2 gram) contained in a gelatin capsule, quickly rinse out the mouth with water, and then test the saliva at once for iodine. This test should be negative. Make additional tests for iodine at 2-minute intervals. The test for iodine is made as follows:

Take 1 c.c. of $NaNO_2$ and 1 c.c. of dilute H_2SO_4[1] in a test-tube, add a little saliva directly from the mouth, and a small amount of starch paste. If convenient, the urine may also be tested. The formation of a blue color signifies that the potassium iodide is being excreted through the salivary glands. Note the length of time elapsing between the ingestion of the potassium iodide and the appearance of the first traces of the substance in the saliva. The chemical reactions taking place in this experiment are indicated in the following equations:

(*a*) $$2NaNO_2 + H_2SO_4 = 2HNO_2 + Na_2SO_4.$$

(*b*) $$2KI + H_2SO_4 = 2HI + K_2SO_4.$$

(*c*) $$2HNO_2 + 2HI = I_2 + 2H_2O + 2NO.$$

20. **Qualitative Analysis of the Products of Salivary Digestion.**—To 25 c.c. of the products of salivary digestion (saved from Experiment 12 or furnished by the instructor), add 100 c.c. of 95 per cent alcohol. Allow to stand until the white precipitate has settled. Filter, evaporate the filtrate to dryness, dissolve the residue in 5–10 c.c. of water and try Fehling's test (p. 27) and the phenylhydrazine reaction (see Dextrose, 3, page 23). On the dextrin precipitate try the iodine test (page 45). Also hydrolyze the dextrin as given under Dextrin, 4, page 48.

[1] Instead of this mixture a few drops of HNO_3 possessing a yellowish or brownish color due to the presence of HNO_2 may be employed.

CHAPTER IV.

PROTEINS:[1] THEIR DECOMPOSITION AND SYNTHESIS.

THE proteins are a class of substances, which in the light of our present knowledge, consist, *in the main*, of combinations of α-amino-acids or their derivatives. These protein substances form the chief constituents of many of the fluids of the body, constitute the organic basis of animal tissue, and at the same time occupy a decidedly pre-eminent position among our organic food-stuffs. They are absolutely necessary to the uses of the animal organism for the continuance of life and they cannot be satisfactorily replaced in the diet of such an organism by any other dietary constituent either organic or inorganic. Such an organism may *exist* without protein food for a period of time, the length of the period varying according to the specific organism and the nature of the substitution offered for the protein portion of the diet. Such a period is, however, distinctly one of *existence* rather than one of normal life and one which is consequently not accompanied by such a full and free exercise of the various functions of the organism as would be possible upon an evenly balanced ration, *i. e.*, one containing the requisite amount of protein food. These protein substances are, furthermore, essential constituents of *all living cells* and therefore without them *vegetable life* as well as animal life is impossible.

The proteins, which constitute such an important group of substances, differ from the carbohydrates and fats very decidedly in elementary composition. In addition to containing *carbon*, *hydrogen*, and *oxygen*, which are present in fats and carbohydrates, the proteins invariably contain *nitrogen* in their molecule and generally *sulphur* also. Proteins have also been identified which contain *phosphorus*, *iron*, *copper*, *iodine*, *manganese*, and *zinc*. The percentage composition of the more important members of the group of protein substances would fall within the following limits: C = 50–55 per cent, H = 6–7.3 per cent, O = 19–24 per cent, N = 15–19 per cent, S = 0.3–2.5 per cent, P = 0.4–0.8 per cent *when present*. When *iron*, *copper*, *iodine*, *manga-*

[1] The term *proteid* has been very widely used by English-speaking scientists to signify the class of substances we have called *proteins*.

nese, or *zinc* are present in the protein molecule they are practically without exception present only in *traces*.[1]

Of all the various elements of the protein molecule, ***nitrogen*** is by far the most important. The human body needs nitrogen for the continuation of life, but it cannot use the nitrogen of the air or that in various other combinations as we find it in nitrates, nitrites, etc. However, in the protein molecule the nitrogen is present in a form which is utilizable by the body. The nitrogen in the protein molecule occurs in at least *four* different forms as follows:

I. Monamino acid nitrogen.
II. Diamino acid nitrogen or *basic* nitrogen.
III. Amide nitrogen.
IV. A guanidine residue.

The actual structure of the protein molecule is still unknown, and we have as yet no means by which its molecular weight can be even approximately established. The many attempts which have been made to determine this have led to very different results, some of which are given in the following table:

Serum albumin	=	4572 – 5100 – 5135
Egg albumin	=	4900 – 6542
Globin	=	15000 – 16086
Oxyhæmoglobin	=	14800 – 15000 – 16655 – 16730

Of these figures, those given for oxyhæmoglobin deserve the most consideration, for these are based on the atomic ratios of the sulphur and iron contained in this substance. The simplest formula that can be calculated from analyses of oxyhæmoglobin, namely, $C_{658}H_{1181}N_{207}S_2FeO_{210}$, serves to show the great complexity of this substance. The following formulas which have been proposed for typical protein substances may serve to further impress the fact of the great size of the protein molecule:

Egg albumin	=	$C_{239}H_{386}N_{58}S_2O_{78}$
Serum albumin	=	$C_{450}H_{720}N_{116}S_6O_{140}$

The decomposition[2] of protein substances may be brought about by oxidation or hydrolysis, but inasmuch as the hydrolytic procedure

[1] Some investigators regard these elements as contaminations, or constituents of some non-protein substance combined with the protein.

[2] The terms "degradation," "dissociation," and "cleavage," are often used in this connection.

has been productive of the more satisfactory results, that type of decomposition procedure alone is used at present. This hydrolysis of the protein molecule may be accomplished by acids, alkalis, or superheated steam, and in digestion by the action of the proteolytic enzymes. The character of the decomposition products varies according to the method utilized in tearing the molecule apart. Bearing this in mind, we may say that the decomposition products of proteins include *proteoses*, *peptones*, *peptides*, *carbon dioxide*, *ammonia*, *hydrogen sulphide*, and *amino acids*. These amino acids constitute a long list of important substances which contain nuclei belonging either to the *aliphatic*, *carbocyclic*, or *heterocyclic* series. The list includes *glycocoll*, *alanine*, *serine*, *phenylalanine*, *tyrosine*, *cystine*, *tryptophane*, *histidine*, *valine*, *arginine*, *leucine*, *isoleucine*, *lysine*, *aspartic acid*, *glutamic acid*, *proline*, *oxyproline*, and *diaminotrihydroxydodecanoic acid*. Of these amino acids, tyrosine and phenylalanine contain carbocyclic nuclei, histidine, proline, and tryptophane contain heterocyclic nuclei, and the remaining members of the list, as given, contain aliphatic nuclei. The amino acids are preëminently the most important class of protein decomposition products. These amino acids are all α-amino acids, and, with the exception of glycocoll, are all optically active. Furthermore, they are amphoteric substances and consequently are able to form salts with both bases and acids. These properties are inherent in the NH_2 and COOH groups of the amino acids.

The decomposition products of protein may be grouped as *primary* and *secondary* decomposition products. By *primary* products are meant those which exist as radicals within the protein molecule and which are liberated, upon cleavage of this molecule, with their carbon chains intact and the position of their nitrogen unaltered. The *secondary* products are those which result from the disintegration of the primary cleavage products. No matter what method is used to decompose a given protein molecule, the primary products are largely the same under all conditions.[1]

In the process of hydrolysis the protein molecule is gradually broken down and less complicated aggregates than the original molecule are formed, which are known as *proteoses*, *peptones*, and *peptides*, and which still possess true protein characteristics. Further hydrolysis causes the ultimate transformation of these substances, of a protein nature, into the amino acids of known chemical structure. In this decomposition the protein molecule is not broken down in a regular

[1] Alkaline hydrolysis yields *urea* and *ornithine* which result from *arginine*, the product of acid hydrolysis.

manner into 1/2, 1/4, 1/8 portions and the amino acids formed *in a group* at the termination of the hydrolysis. On the contrary, certain amino acids are formed very early in the process, in fact while the main hydrolytic action has proceeded no further than the proteose stage. Gradually the complexity of the protein portion undergoing decomposition is simplified by the splitting off of the amino acids and finally it is so far decomposed through previous cleavages that it yields only amino acids at the succeeding cleavage. In short, the general plan of the hydrolysis of the protein molecule is similar to the hydrolysis of starch. In the case of starch there is formed a series of dextrins of gradually decreasing complexity and coincidently with the formation of each dextrin a small amount of sugar is split off and finally nothing but sugar remains. In the case of protein hydrolysis there is a series of proteins of gradually decreasing complexity produced and coincidently with the formation of each new protein substance amino acids are split off and finally the sole products remaining are amino acids.

Inasmuch as diversity in the method of decomposing a given protein does not result in an equally diversified line of decomposition products, but, on the other hand, yields products which are quite comparable in character, it may be argued that there are probably well-defined lines of cleavage in the individual protein molecule and that no matter what the force brought to bear to tear such a molecule apart, the disintegration, when it comes, will yield in every case certain definite fragments. These fragments may be called the "building stones" of the protein molecule, a term used by some of the German investigators. Take, for example, the decomposition of protein which may be brought about through the action of the enzyme *trypsin* of the pancreatic juice. When this enzyme is allowed to act upon a given protein, the latter is disintegrated in a series of definite cleavages, resulting in the formation of *proteoses*, *peptones*, and *peptides* in regular order, the peptides being the last of the decomposition products which possess protein characteristics. They are all built up from amino acids and are therefore closely related to these acids on the one side and to peptones on the other. We have *di-*, *tri-*, *tetra-*, *penta-*, *deca-*, and *poly-peptides* which are named according to the number of amino acids included in the peptide molecule. Following the peptides there are a diverse assortment of *monamino* and *diamino* acids which constitute the final products of the protein decomposition. These acids are devoid of any protein characteristics and are therefore decidedly different from the original substance from which they were derived. From a protein of huge molecular weight, a typical colloid, perhaps

but slightly soluble, and entirely non-diffusible, we have passed by way of proteoses, peptones, and peptides to a class of simpler crystalline substances which are, for the most part, readily soluble and diffusible.

These amino acids after their production in the process of digestion, as just indicated, are synthesized within the organism to form protein material which goes to build up the tissues of the body. It is thus seen that the amino acids are of prime importance in the animal economy. Moreover, it is important to remember that these essential factors in metabolism and nutrition cannot be produced within the animal organism from their elements, but are only yielded upon the hydrolysis of ingested protein of animal or vegetable origin.

When we examine the formulas of the principal members of the crystalline end-products of protein decomposition we note that they are invariably acids, as has already been mentioned, and contain an NH_2 group in the α position. This relation of the NH_2 group to the acid radical is *constant*, no matter what other groups or radicals are present. We may have straight *chains* as in alanine and glutamic acid, the *benzene ring* as in phenylalanine, or we may have *sulphurized bodies* as in cystine and still the formula is always of the same type, *i. e.*,

$$\begin{array}{l} NH_2 \\ | \\ R{-}CH{-}COOH \end{array}$$

It is seen that this characteristic grouping in the amino acid provides each one of these ultimate fragments of the protein molecule with both a strong *acid* and a strong *basic* group. For this reason it is theoretically possible for a large number of these amino acids to combine and the resulting combinations may be very great in number, since there is such a varied assortment of the acids. The protein molecule, which is of such mammoth proportions, is probably constructed on a foundation of this sort. Of late much valuable data have been collected regarding the synthetic production of protein substances, the leaders in this line of investigation being Fischer and Abderhalden. After having gathered a mass of data regarding the final products of the protein decomposition and demonstrating that amino acids were the ultimate results of the various forms of decomposition, these investigators, and notably Fischer, set about in an effort to form, from these amino acids, by synthetic means, substances which should possess protein characteristics. The simplest of these

bodies formed in this way was synthesized from two molecules of glycocoll with the liberation of water, thus:

$$CH_2—NH_2—CO\ OH\ H\ HN—CH_2—COOH.$$

The body thus formed is a *dipeptide*, called *glycyl-glycine*. In an analogous manner may be produced *leucyl-leucine*, through the synthesis of two molecules of leucine or *leucyl-alanyl-glycine* through the union of one molecule of leucine, one of alanine, and one of glycocoll. By this procedure Fischer and his pupils have been able to make a large number of peptides containing varied numbers of amino-acid radicals, the name *polypeptides* being given to the whole group of synthetic substances thus formed. The most complex polypeptide yet produced is one containing fifteen glycocoll and three leucine residues.

Notwithstanding the fact that most synthetic polypeptides are produced through a union of amino acids by means of their imide bonds, it must not be imagined that the protein molecule is constructed from amino acids linked together in straight chains in a manner analogous to the formation of simple peptides, such as glycyl-glycine. The molecular structure of the proteins is much too complex to be explained upon any such simple formation as that. There must be a variety of linkings, since there is a varied assortment of decomposition products of totally different structure.

Many of these synthetic bodies respond to the biuret test, are precipitated by phosphotungstic acid, and behave, in other ways, as to leave no doubt as to their protein characteristics. For instance, a number of amino acids each possessing a *sweet* taste have been synthesized in such a manner as to yield a polypeptide of *bitter* taste, a well known characteristic of peptones. From the fact that the polypeptides formed in the manner indicated have free acidic and basic radicals we gather the explanation of the amphoteric character of true proteins. Fischer expresses the encouraging belief that he will soon be able to produce true protein by the synthesis of its decomposition products. *Silk fibroin* is the protein substance he expects to synthesize. He no doubt will perform this joint office for *organic* and *physiological* chemistry if it is capable of performance by the present methods of technique. Even Fischer, however, is frank enough to say that the production of the great body of protein substances synthetically, will, under the most encouraging conditions, be a *terrific task*, involving the "life-work of a whole army of inventive and diligent chemists."

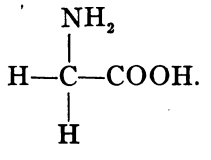
For the benefit of those especially interested in such matters a photograph of the Fischer apparatus (Fig. 22, page 70) used in the fractional distillation, *in vacuo*, of the esters of the decomposition products of the proteins, as well as micro-photographs and drawings of preparations of several of these decomposition products (Figs. 19 to 31, pages 67 to 79) are introduced. For the preparations and the photograph of the apparatus the author is indebted to Dr. T. B. Osborne, of New Haven, Conn., who has made many important observations upon the hydrolysis of proteins. The reproduction of the crystalline form of some of the more recent of the products may be of interest to those viewing the field of physiological chemistry from other than the student's aspect.

An extended discussion of the various decomposition products being out of place in a book of this character, we will simply make a few general statements in connection with the primary decomposition products.

DISCUSSION OF THE PRODUCTS.

Ammonia, NH_3.—Ammonia is an important decomposition product of all proteins and probably arises from an amide group combined with a carboxyl group of some of the amino acids. It is possible that the dibasic acids, aspartic and glutamic, furnish most of these carboxyl groups. This is indicated by the more or less close relationship which exists between the amount of ammonia and that of the dibasic acids which the several proteins yield upon decomposition. The elimination of the ammonia from proteins under the action of acids and alkalis is very similar to that from amides like asparagine.

Glycocoll, $C_2H_5NO_2$.—Glycocoll, or *amino acetic acid,* is the simplest of the amino acids and has the following formula:



Glycocoll, as the formula shows, contains no asymmetric carbon atom, and is the only amino acid yielded by protein decomposition which is *optically inactive.* Glycocoll and leucine were the first decomposition products of proteins to be discovered (1820). Upon administering benzoic acid to animals the output of hippuric acid in the urine is greatly increased, thus showing a synthesis of benzoic

acid and glycocoll in the organism (see p. 157, Chapter IX). Glycocoll, ingested in small amount, is excreted in the urine as urea, whereas if administered in excess it appears in part unchanged in the urine. It is usually separated from the mixture of protein decomposition products as the hydrochloride of the ester. The crystalline form of this compound is shown in Fig. 19.

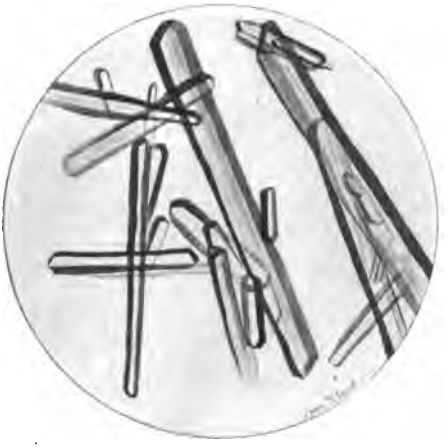
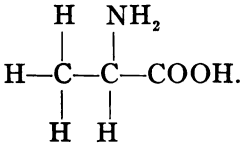


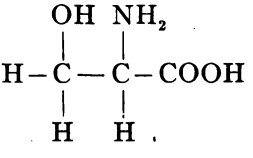
FIG. 19.—GLYCOCOLL ESTER HYDROCHLORIDE.

Alanine, $C_3H_7NO_2$.—Alanine is *α-amino-propionic acid*, and as such it may be represented structurally as follows:



Obtained from protein substances, alanine is dextro-rotatory, is very soluble in water, and possesses a sweet taste. Tyrosine, phenylalanine, cystine, and serine are derivatives of alanine. This amino acid has been obtained from nearly all proteins examined. Its absence from those proteins from which it has not been obtained has not been proven. Most proteins yield relatively small amounts of alanine.

Serine, $C_3H_7NO_3$.—Serine is *α-amino-β-hydroxy-propionic acid* and possesses the following structural formula:



Serine obtained from proteins is lævo-rotatory, possesses a sweet taste, and is quite soluble in water. Serine is not obtained in quantity from most proteins, but is yielded abundantly by *silk glue*. Owing to the difficulty of separating serine it has not been found in a number of proteins in which it probably occurs. Serine crystals are shown in Fig. 20, below.

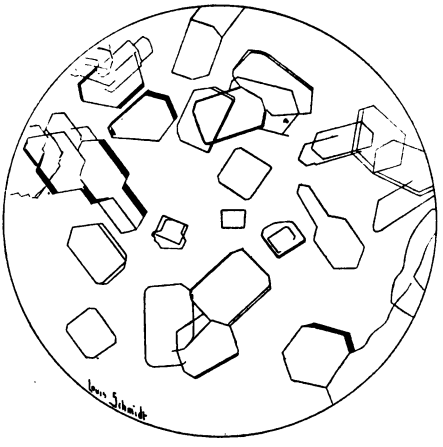
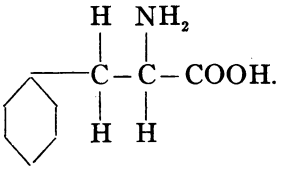


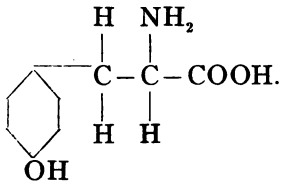
FIG. 20.—SERINE.

Phenylalanine, $C_9H_{11}NO_2$.—This product is *phenyl-α-amino-propionic acid*, and may be represented graphically as follows:



The lævo-rotatory form is obtained from proteins. Phenylalanine has been obtained from all the proteins examined except from the protamines and some of the albuminoids. The yield of this body from the decomposition of proteins is frequently greater than the yield of tyrosine. The crystalline form of phenylalanine is shown in Fig. 21, p. 69.

Tyrosine, $C_9H_{11}NO_3$.—Tyrosine, one of the first discovered end-products of protein decomposition, is the amino acid, *p-oxyphenyl-α-amino-propionic acid*. It has the following formula:



The tyrosine which results from protein decomposition is usually lævo-rotatory although the dextro-rotatory form sometimes occurs. Tyrosine is one of the end-products of tryptic digestion and usually

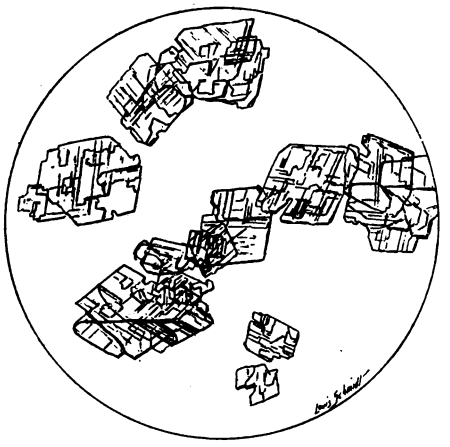


FIG. 21.—PHENYLALANINE.

separates in conspicuous amount early in the process of digestion. It does not occur, however, as an end-product of the decomposition of gelatin.

Tyrosine is found in old cheese, and derives its name from this fact. It crystallizes in tufts, sheaves, or balls of fine needles, which decompose at 295° C. and are sparingly soluble in cold (1–2454) water, but much more so in boiling (1–154) water. Tyrosine forms soluble salts with alkalis, ammonia, or mineral acids, and is soluble, with difficulty, in acetic acid. It responds to Millon's reaction, thus showing the presence of the hydroxyphenyl group, but gives no other protein test. The aromatic groups present in tyrosine, phenyl-alanine, and tryptophane cause proteins to yield a positive xanthoproteic reaction. In severe cases of typhoid fever and smallpox, in acute yellow atrophy of the liver, and in acute phosphorus poisoning, tyrosine has been found in the urine. Tyrosine crystals are shown in Fig. 23, p. 71.

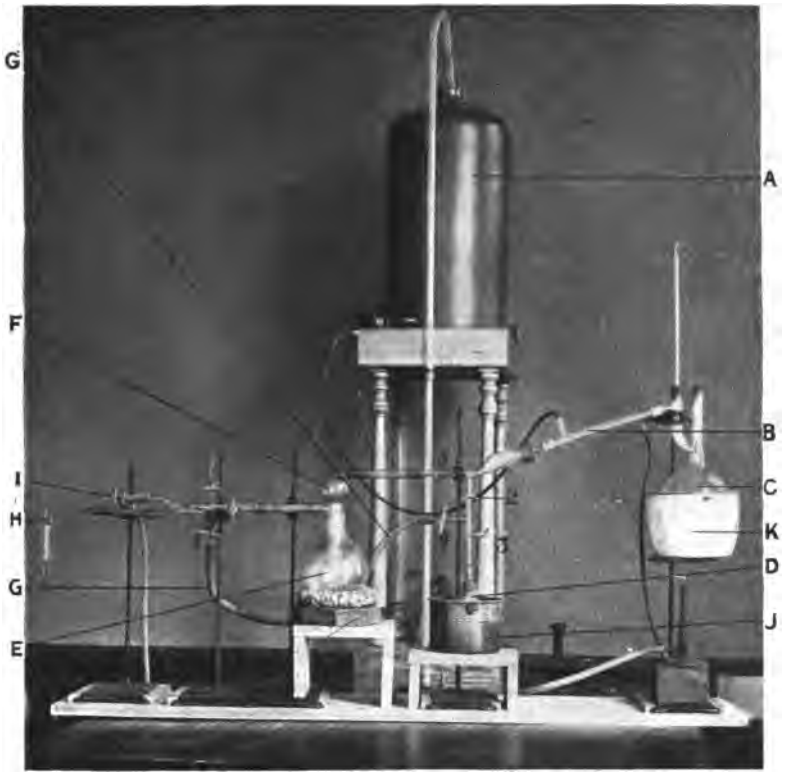
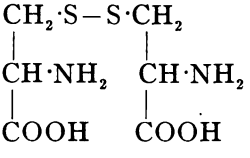


FIG. 22.—FISCHER APPARATUS.

Reproduced from a photograph made by Prof. E. T. Reichert, of the University of Pennsylvania. The negative was furnished by Dr. T. B. Osborne, of New Haven, Conn.

A, Tank into which freezing mixture is pumped and from which it flows through the condenser, *B; C*, flask from which the esters are distilled, the distillate being collected in *D; E*, a Dewar flask containing liquid air serving as a cooler for condensing tube *F; G* and *G'*, tubes leading to the Geryck pump by which the vacuum is maintained; *I*, tube leading to a McLeod gauge (not shown in figure); *J*, a bath containing freezing mixture in which the receiver *D* is immersed; *K*, a bath of water during the first part of the distillation and of oil during the last part of the process; 1–5, stop cocks which permit the cutting out of different parts of the apparatus as the procedure demands.

Cystine, $C_6H_{12}O_4N_2S_2$.—Friedmann has recently shown cystine to be the *disulphide of α-amino-β-thiolactic acid*[1] and to possess the following structural formula:



[1] Also called α-diamino-β-dithio-dilactylic acid.

Cystine is the principal sulphur-containing body obtained from the decomposition of protein substances. It is obtained in greatest amount as a decomposition product of such keratin-containing tissues

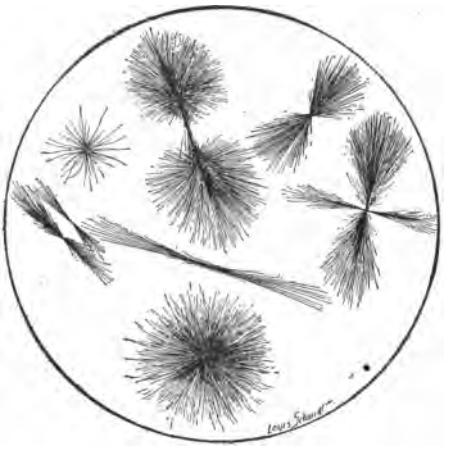


FIG. 23.—TYROSINE.

as horn, hoof, and hair. Cystine occurs in small amount in normal urine and is greatly increased in quantity under certain pathological conditions. It crystallizes in thin, colorless, hexagonal plates which

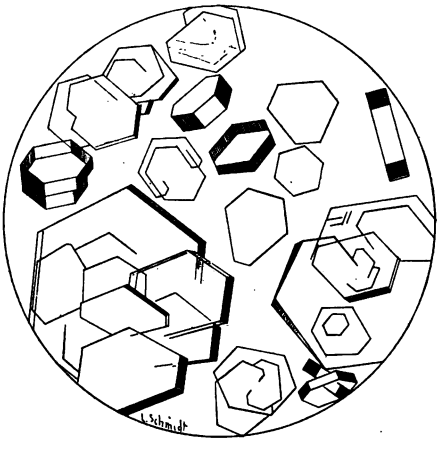


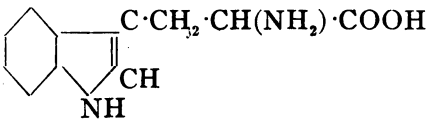
FIG. 24.—CYSTINE.

are shown in Fig. 24. Cystine is very *slightly* soluble in water but its salts, with both bases and acids, are *readily* soluble in water. It is lævo-rotatory.

It has recently been claimed that cystine occurs in two forms, *i. e.*, stone-cystine and protein-cystine and that these two forms are distinct in their properties. This view is incorrect.

For a discussion of cystine sediments in urine see Chapter XX.

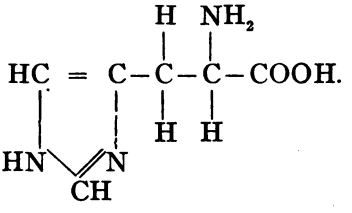
Tryptophane, $C_{11}H_{12}N_2O_2$.—According to Ellinger, tryptophane is *indol-α-amino-propionic acid.* Recently Ellinger and Flamand have shown that it possesses the following formula:



Tryptophane is the *mother-substance of indole, skatole, skatole acetic acid* and *skatole carboxylic acid,* all of which are formed as *secondary* decomposition products of proteins. Its presence in protein substances may be shown by means of the Adamkiewicz reaction or the Hopkins-Cole reaction (see page 89). It may be detected in a tryptic digestion mixture through its property of giving a violet color-reaction with bromine water. Tryptophane is yielded by nearly all proteins, but has been shown to be *entirely absent from zein,* the prolamin (alcohol-soluble protein) of maize.

Solutions of tryptophane in sodium hydroxide are dextro-rotatory. Upon being heated to 266° C. tryptophane decomposes with the evolution of gas.

Histidine, $C_6H_9N_3O_2$.—Histidine is *α-amino-β-imidazol-propionic acid* with the following structural formula:



The histidine obtained from proteins is lævo-rotatory. It has been obtained from all the proteins thus far examined, the majority of them yielding about 2.5 per cent of the amino acid. However, about 11 per cent was obtained by Abderhalden from *globin,* the protein constituent of oxyhæmoglobin and about 13 per cent by Kossel and Kutscher from the protamine *sturine.*

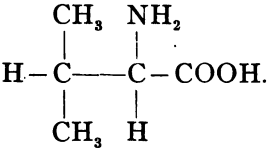
Crystals of histidine dichloride are shown in Fig. 25, below.

Knoop's Color Reaction for Histidine.—To an aqueous solution of histidine or a histidine salt in a test-tube add a little bromine water. A yellow coloration develops in the cold and upon further addition of bromine water becomes permanent. If the tube be heated,[1] the color will disappear and will shortly be replaced by a faint red coloration which gradually passes into a deep wine red. Usually black, amorphous particles separate out and the solution becomes turbid.

FIG. 25.—HISTIDINE DICHLORIDE.

The reaction cannot be obtained in solutions containing free alkali. It is best to use such an amount of bromine as will produce a permanent yellow color in the cold. The use of a less amount of bromine than this produces a weak coloration whereas an excess of bromine prevents the reaction. The test is not very delicate, but a characteristic reaction may always be obtained in 1 : 1000 solutions. The only histidine derivative which yields a similar coloration is imidazolethylamine, and the reaction in this case is rather weak as compared with the color obtained with histidine or histidine salts.

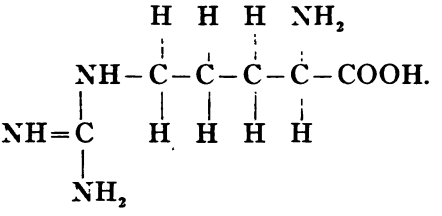
Valine, $C_5H_{11}NO_2$.—The amino-valerianic acid obtained from proteins is *α-amino-isovalerianic acid,* and as such bears the following formula:



[1] The same reaction will take place in the cold more slowly.

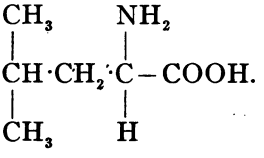
It closely resembles leucine in many of its properties, but is more soluble in water. It is a difficult matter to identify valine in the presence of leucine and isoleucine inasmuch as these amino acids crystallize together in such a way that the combination persists even after repeated recrystallizations. Valine is dextro-rotatory.

Arginine, $C_6H_{14}N_4O_2$.—Arginine is *guanidine-α-amino-valerianic acid* and possesses the following structural formula:



It has been obtained from every protein so far subjected to decomposition. The arginine obtained from proteins is dextro-rotatory, and has pronounced basic properties, reacts strongly alkaline to litmus, and forms stable carbonates. Because of these facts, some investigators consider arginine to be the nucleus of the protein molecule. It is obtained in widely different amounts from different proteins, over 85 per cent of certain protamines having been obtained in the form of this amino acid. It is claimed that in the ordinary metabolic activities of the animal body arginine gives rise to urea. While this claim is probably true, it should, at the same time, be borne in mind that the greater part of the protein nitrogen is eliminated as urea and that, therefore, but a very small part can arise from arginine.

Leucine, $C_6H_{13}NO_2$.—Leucine is an abundant end-product of the decomposition of protein material, and, together with glycocoll, was the first of these products to be discovered (1820). It is *α-amino-isobutyl-acetic acid*, and therefore has the following formula:



The leucine which results from protein decomposition is *l*-leucine. Leucine is present *normally* in the pancreas, thymus, thyroid, spleen, brain, liver, kidneys, and salivary glands. It has been found *pathologically* in the urine (in acute yellow atrophy of the liver, in acute phosphorus poisoning, and in severe cases of typhoid fever and smallpox), and in the liver, blood, and pus.

Pure leucine crystallizes in thin, white, hexagonal plates. Crystals of pure leucine are reproduced in Fig. 26. It is rather easily soluble in water (46 parts), alkalis, ammonia, and acids. On rapid heating to 295° C., leucine decomposes with the formation of carbon dioxide, ammonia, and amylamine. Aqueous solutions of leucine obtained from proteins are lævo-rotatory, but its acid or alkaline solutions are dextro-rotatory. So-called impure leucine[1] is a slightly refractive

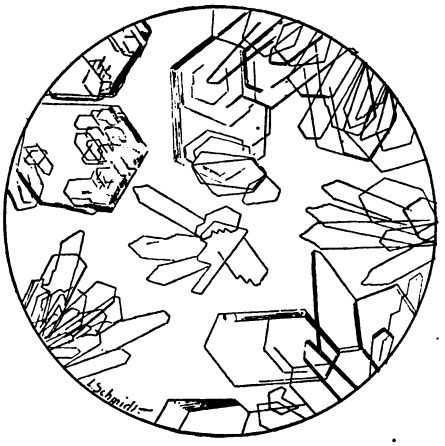
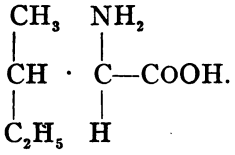


FIG. 26.—LEUCINE.

substance, which generally crystallizes in balls having a radial structure, or in aggregations of spherical bodies, Fig. 104, Chapter XX.

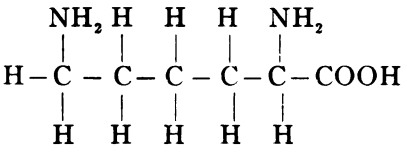
Isoleucine, $C_6H_{13}NO_2$.—Isoleucine is *α-amino-methyl-ethyl-propionic acid*, and possesses the following structural formula:



This amino acid was recently discovered by Ehrlich. Its presence has been established among the decomposition products of only a few proteins although it probably occurs among those of many or most of them. Ehrlich has shown that the *d*-amyl alcohol which is produced by yeast fermentation originates from isoleucine and the isoamylalcohol originates from leucine. Isoleucine is dextro-rotatory.

[1] These balls of so-called *impure* leucine do contain considerable leucine, but inasmuch as they may contain many other things it is a bad practice to allude to them as *leucine*.

Lysine, $C_6H_{14}N_2O_2$.—The three bodies, lysine, arginine, and histidine, are frequently classed together as the *hexone bases*. Lysine was the first of the bases discovered. It is *α-ε-diamino-caproic acid* and hence possesses the following structure:

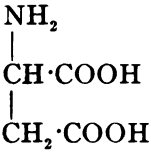


It is dextro-rotatory and is found in relatively large amount in casein and gelatin. Lysine is obtained from nearly all proteins, but is absent from the vegetable proteins which are soluble in strong alcohol. It

FIG. 27.—LYSINE PICRATE.

is the mother-substance of cadaverin and has never been obtained in crystalline form. Lysine is usually obtained as the picrate which is sparingly soluble in water and crystallizes readily. These crystals are shown in Fig. 27.

Aspartic Acid, $C_4H_7NO_4$.—Aspartic acid is *amino-succinic acid* and has the following structural formula:



The amide of aspartic acid, *asparagine*, is very widely distributed in the vegetable kingdom. The crystalline form of aspartic acid is exhibited in Fig. 28.

Aspartic acid has been found among the decomposition products of all the proteins examined, *except the protamines*. It has not been obtained, however, in very large proportion from any of them. The aspartic acid obtained from protein is lævo-rotatory.

FIG. 28.—ASPARTIC ACID.

Glutamic Acid, $C_5H_9NO_4$.—This acid is *α-amino-normal-glutaric acid* and as such bears the following graphic formula:

$$\begin{array}{l} NH_2 \\ | \\ CH\cdot COOH \\ | \\ CH_2 \\ | \\ CH_2\cdot COOH. \end{array}$$

Glutamic acid is yielded by all the proteins thus far examined, except the protamines, and by most of these in larger amount than any other of their decomposition products. It is yielded in especially large proportion by most of the proteins of seeds, 41.32 per cent having been obtained very recently by Kleinschmitt from the hydrolysis of *hordein*, the prolamin of barley. This is the largest amount of any single decomposition product yet obtained from any protein except the protamines.[1]

[1] Up to this time the yield of 37.33 per cent obtained by Osborne and Harris from gliadin of wheat was the maximum yield.

Glutamic acid and aspartic acid are the only dibasic acids which have thus far been obtained as decomposition products of proteins. As there is an apparent relation between the proportion of these acids and that of ammonia which the different proteins yield it is possible that one of the carboxyl groups of these acids is united with NH_2 as an amide, the other carboxyl group being united in poly-

FIG. 29.—GLUTAMIC ACID.
Reproduced from a micro-photograph made by Prof. E. T. Reichert, of the University of Pennsylvania.

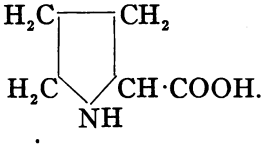
peptide union (see page 64) with some other amino acid. This might be represented by the following formula:

$$\begin{array}{l} R-CHNH-COOH \\ \quad\quad\quad\quad\quad | \\ \quad\quad CO-CHNH_2-CH_2-CH_2-CONH_2. \end{array}$$

It has not been definitely proven, however, that this form of linking actually occurs.

The glutamic acid, yielded by proteins upon hydrolysis, is dextro-rotatory. Crystals of glutamic acid are reproduced in Fig. 29, above.

Proline, $C_5H_9NO_2$.—Proline is *α-pyrrolidine-carboxylic acid* and possesses the following graphic structure:



Proline was first obtained as a decomposition product of casein. Proline obtained from proteins is lævo-rotatory and is the only protein decomposition product which is readily soluble in alcohol. It is also

one of the few heterocyclic compounds obtained from proteins. Proline was quite recently discovered, but has since been found among the decomposition products of all proteins except the protamines.

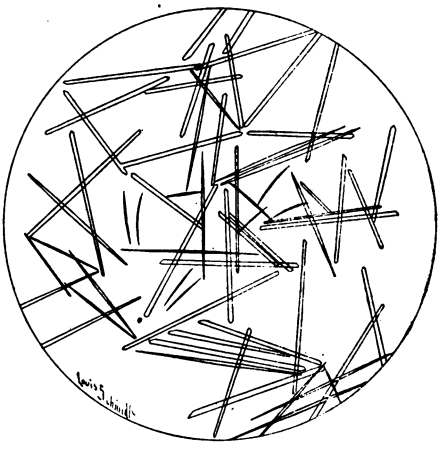


FIG. 30.—LÆVO-α-PROLINE.

The maximum yield reported is 13.73 per cent obtained by Osborne and Clapp from the hydrolysis of hordein. More recently Fischer and Boehner[1] reported having obtained 7.7 per cent from the hydrolysis

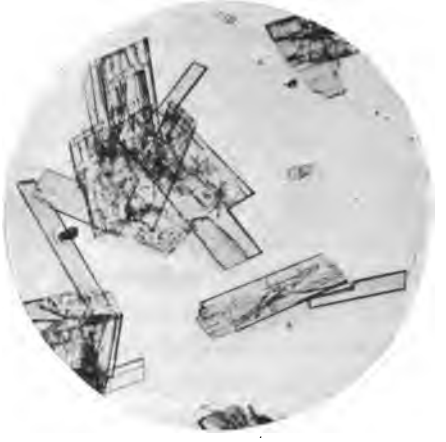


FIG. 31.—COPPER SALT OF PROLINE.
Reproduced from a micro-photograph made by Prof. E. T. Reichert, of the University of Pennsylvania.

of gelatine. The crystalline form of *lævo-α-proline* is shown in Fig. 30, and the copper salt of proline is represented by a micro-photograph in Fig. 31, above. The crystals of the copper salt have a deep blue color,

[1] Fischer and Boehner: *Zeit. phys. chem.*, 65, p. 118, 1910.

but when they lose their water of crystallization they assume a characteristic violet color.

Oxyproline, $C_5H_9NO_3$.—Oxyproline was recently discovered by Fischer. It has as yet been obtained from only a few proteins, but this may be due to the fact that only a few have been examined for its presence. Its structure has not yet been established.

Diaminotrihydroxydodecanoic Acid, $C_{12}H_{26}N_2O_5$.—This amino acid was discovered by Fischer and Abderhalden as a product of the hydrolysis of casein. It has thus far been obtained from no other source. It is lævo-rotatory and its constitution has not been determined.

EXPERIMENTS.

While the ordinary courses in physiological chemistry preclude any extended study of the decomposition products of proteins, the manipulation of a simple decomposition and the subsequent isolation and study of a few of the products most easily and quickly obtained will not be without interest.[1] To this end the student may use the following decomposition procedure: Treat the protein in a large flask with water containing 3–5 per cent of H_2SO_4 and place it on a water-bath until the protein material has been decomposed and there remains a fine, fluffy, insoluble residue. Filter off this residue and neutralize the filtrate with $Ba(OH)_2$ and $BaCO_3$. Filter off the precipitate of $BaSO_4$ which forms and when certain that the fluid is neutral or faintly acid,[2] concentrate (first on a wire gauze and later on a water-bath) to a syrup. This syrup contains the end-products of the decomposition of the protein, among which are *proteoses*, *peptones*, *tyrosine*, *leucine*, etc. Add 95 per cent alcohol slowly to the warm syrup until no more precipitate forms, stirring continuously with a glass rod. This precipitate consists of proteoses and peptones. Gather the sticky precipitate on the rod or the sides of the dish, and, after warming the solution gently for a few moments, filter it through a filter paper which has not been previously moistened. After dissolving the precipitate of proteoses and peptones in water[3]

[1] The procedure here set forth has nothing in common with the procedure by means of which the long line of decomposition products just enumerated are obtained. This latter process is an exceedingly complicated one which is entirely outside the province of any course in physiological chemistry.

[2] If the solution is alkaline in reaction at this point, the amino acids will be broken down and ammonia will be evolved.

[3] At this point the aqueous solution of the proteoses and peptones may be filtered to remove any $BaSO_4$ which may still remain. Tyrosine crystals will also be found here, since it is less soluble than the leucine and may adhere to the proteose-peptone precipitate. Add the crystals of tyrosine to the warm alcohol filtrate.

the solution may be treated according to the method of separation given on page 112.

The leucine and tyrosine, etc., are in solution in the warm alcoholic filtrate. Concentrate this filtrate on the water-bath to a thin syrup, transfer it to a beaker, and allow it to stand over night in a cool place for crystallization. The tyrosine first crystallizes (Fig. 23, page 71), followed later by the formation of characteristic crystals of impure leucine (see Fig. 105, Chapter XX). After examining these crystals under the microcsope, strain off the crystalline material through fine muslin, heat it gently in a little water to dissolve the leucine (the tyrosine will be practically insoluble) and filter. Concentrate the filtrate and allow it to stand in a cool place over night for the crude leucine to crystallize. Filter off the crystals and use them in the tests for leucine given on page 82. The crystals of tyrosine remaining on the paper from the first filtration may be used in the tests for tyrosine as given below. If desired, the tyrosine and leucine may be purified by recrystallizing in the usual manner. Habermann has suggested a method of separating leucine and tyrosine by means of glacial acetic acid.

Experiments on Tyrosine.

Make the following tests with the tyrosine crystals already prepared or upon some pure tyrosine furnished by the instructor.

1. **Microscopical Examination.**—Place a minute crystal of tyrosine on a slide, add a drop of water, cover with a coverglass, and examine microscopically. Now run more water under the coverglass and warm in a bunsen flame until the tyrosine has dissolved. Allow the solution to cool *slowly*, then examine again microscopically, and compare the crystals with those shown in Fig. 23, page 71.

2. **Solubility.**—Try the solubility of very *small amounts* of tyrosine in cold and hot water, cold and hot 95 per cent alcohol, dilute NH_4OH, dilute KOH and dilute HCl.

3. **Sublimation.**—Place a little tyrosine in a *dry* test-tube, heat gently and notice that the material does not sublime. How does this compare with the result of Experiment 3 under Leucine?

4. **Hoffman's Reaction.**—This is the name given to Millon's reaction when employed to detect tyrosine. Add about 3 c.c. of water and a few drops of Millon's reagent to a little tyrosine in a test-tube. Upon dissolving the tyrosine by heat the solution gradually darkens and may assume a dark red color. What group does this test show to be present in tyrosine?

5. **Piria's Test.**—Warm a little tyrosine on a watch glass on a boiling water-bath for 20 minutes with 3–5 drops of conc. H_2SO_4. Tyrosine sulphuric acid is formed in the process. Cool the solution and wash it into a small beaker with water. Now add $CaCO_3$ in substance slowly with stirring, until the reaction of the solution is no longer acid. Filter, concentrate the filtrate, and add to it a few drops (avoid an excess) of very dilute neutral ferric chloride. A purple or violet color, due to the formation of the ferric salt of tyrosine-sulphuric acid, is produced. This is one of the most satisfactory tests for the identification of tyrosine.

6. **Mörner's Test.**—Add about 3 c.c. of Mörner's reagent[1] to a little tyrosine in a test-tube, and *gently* raise the temperature to the boiling-point. A green color results.

Experiments on Leucine.

Make the following tests upon the leucine crystals already prepared or upon some pure leucine furnished by the instructor.

1, 2 and 3. Repeat these experiments according to the directions given under Tyrosine (page 81).

[1] Mörner's reagent is prepared by thoroughly mixing 1 volume of formalin, 45 volumes of distilled water, and 55 volumes of concentrated sulphuric acid.

CHAPTER V.

PROTEINS: THEIR CLASSIFICATION AND PROPERTIES.

FROM what has already been said in Chapter IV regarding the protein substances it will be recognized that the grouping of the diverse forms of this class of substances in a logical manner is not an easy task. The fats and carbohydrates may be classified upon the fundamental principles of their stereo-chemical relationships, whereas such a system of classification in the case of the proteins is absolutely impossible since, as we have already stated, the molecular structure of these complex substances is unknown. Because of the diversity of standpoint from which the proteins may be viewed, relative to their grouping in the form of a logically classified series, it is obvious that there is an opportunity for the presentation of classifications of a widely divergent character. The fact that there were until recently at least a dozen different classifications which were recognized by various groups of English-speaking investigators emphasizes the difficulties in the way of the individual or individuals who would offer a classification which should merit universal adoption. Realizing the great handicap and disadvantage which the great diversity of the protein classifications was forcing upon the workers in this field, the Chemical and Physiological Societies of England recently drafted a classification which appealed to these groups of scientists as fulfilling all requirements and presented it for the consideration of the American Physiological Society and the American Society of Biological Chemists. The outcome of this has been that there are now only *two* protein classifications which are recognized by English-speaking scientists, one the British Classification, the other the American Classification. These classifications are very similar and doubtless will ultimately be merged into a single classification. In our consideration of the proteins we shall conform in all details to the American Classification. In this connection we will say, however, that we feel that the English Societies have strong grounds for preferring the use of the term *scleroproteins* for albuminoids and *chromoproteins* for hæmoglobins. The two classifications are as follows:

CLASSIFICATION OF PROTEINS ADOPTED BY THE AMERICAN PHYSIOLOGICAL SOCIETY AND THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

I. SIMPLE PROTEINS.

Protein substances which yield *only α-amino acids* or their derivatives on hydrolysis.

(*a*) **Albumins.**—Soluble in pure water and coagulable by heat, *e. g.*, *ovalbumin*, *serum albumin*, *lactalbumin*, *vegetable albumins*.

(*b*) **Globulins.**—Insoluble in pure water but soluble in neutral solutions of salts of strong bases with strong acids,[1] *e. g.*, *serum globulin*, *ovoglobulin*, *edestin*, *amandin*, and other *vegetable globulins*.

(*c*) **Glutelins.**—Simple proteins insoluble in *all neutral solvents*, but readily soluble in very dilute acids and alkalis,[2] *e. g.*, *glutenin*.

(*d*) **Alcohol-soluble Proteins (Prolamins).**[3]—Simple proteins soluble in 70–80 per cent alcohol, insoluble in water, absolute alcohol, and other neutral solvents,[4] *e. g.*, *zein*, *gliadin*, *hordein*, and *bynin*.

(*e*) **Albuminoids.**—Simple proteins possessing a similar structure to those already mentioned, but characterized by a pronounced insolubility in all neutral solvents,[5] *e. g.*, *elastin*, *collagen*, *keratin*.

(*f*) **Histones.**—Soluble in water and insoluble in very dilute ammonia, and, in the absence of ammonium salts, insoluble even in excess of ammonia; yield precipitates with solutions of other proteins and a coagulum on heating which is easily soluble in very dilute acids. On hydrolysis they yield a large number of amino acids among which the basic ones predominate. In short, histones are basic proteins which stand between protamines and true proteins, *e. g.*, *globin*, *thymus histone*, *scombrone*.

(*g*) **Protamines.**—Simpler polypeptides than the proteins included in the preceding groups. They are soluble in water, uncoagulable by heat, have the property of precipitating aqueous solutions of other proteins, possess strong basic properties and form stable

[1] The precipitation limits with ammonium sulphate should not be made a basis for distinguishing the albumins from the globulins.

[2] Such substances occur in abundance in the seeds of cereals and doubtless represent a well-defined natural group of simple proteins.

[3] The name *prolamins* has been suggested for these alcohol-soluble proteins by Dr. Thomas B. Osborne (*Science*, 1908, XXVIII, p. 417). It is a very fitting term inasmuch as upon hydrolysis they yield particularly large amounts of *proline* and *ammonia*.

[4] The subclasses defined (*a*, *b*, *c*, *d*,) are exemplified by proteins obtained from both plants and animals. The use of appropriate prefixes will suffice to indicate the origin of the compounds, *e. g.*, ovoglobulin, lactalbumin, etc.

[5] These form the principal organic constituents of the skeletal structure of animals and also their external covering and its appendages. This definition does not provide for gelatin which is, however, an artificial derivative of collagen.

salts with strong mineral acids. They yield comparatively few amino acids, among which the basic ones predominate. They are the *simplest natural proteins, e. g., salmine, sturine, clupeine, scombrine.*

II. CONJUGATED PROTEINS.

Substances which contain the protein molecule united to some other molecule or molecules otherwise than as a salt.

(*a*) **Nucleoproteins.**—Compounds of one or more protein molecules with nucleic acid, *e. g., cytoglobulin, nucleohistone.*

(*b*) **Glycoproteins.**—Compounds of the protein molecule with a substance or substances containing a carbohydrate group other than a nucleic acid, *e. g., mucins* and *mucoids* (*osseomucoid, tendomucoid, ichthulin, helicoprotein*).

(*c*) **Phosphoproteins.**—Compounds of the protein molecule with some, as yet undefined, phosphorus-containing substances other than a nucleic acid or lecithin,[1] *e. g., caseinogen, vitellin.*

(*d*) **Hæmoglobins.**—Compounds of the protein molecule with hæmatin, or some similar substance, *e. g., hæmoglobin, hæmocyanin.*

(*e*) **Lecithoproteins.**—Compounds of the protein molecule with *lecithins, e. g., lecithans, phosphatides.*

III. DERIVED PROTEINS.

1. Primary Protein Derivatives.

Derivatives of the protein molecule apparently formed through hydrolytic changes which involve only slight alteration of the protein molecule.

(*a*) **Proteans.**—Insoluble products which apparently result from the incipient action of water, very dilute acids or enzymes, *e. g., uyosan, edestan.*

(*b*) **Metaproteins.**—Products of the further action of acids and alkalis whereby the molecule is so far altered as to form products soluble in very weak acids and alkalis but insoluble in neutral fluids, *e. g., acid metaprotein* (*acid albuminate*), *alkali metaprotein* (*alkali albuminate*).

(*c*) **Coagulated Proteins.**—Insoluble products which result from (1) the action of heat on their solutions, or (2) the action of alcohol on the protein.

[1] The accumulated chemical evidence distinctly points to the propriety of classifying the phosphoproteins as conjugated compounds, *i. e.*, they are possibly esters of some phosphoric acid or acids and protein.

2. Secondary Protein Derivatives.[1]

Products of the further hydrolytic cleavage of the protein molecule.

(*a*) **Proteoses.**—Soluble in water, non-coagulable by heat, and precipitated by saturating their solutions with ammonium—or zinc sulphate,[2] *e. g.*, *protoproteose, deuteroproteose.*

(*b*) **Peptones.**—Soluble in water, non-coagulable by heat, but *not precipitated* by saturating their solutions with ammonium sulphate,[3] *e. g.*, *antipeptone, amphopeptone.*

(*c*) **Peptides.**—Definitely characterized combinations of two or more amino acids, the *carboxyl* group of one being united with the *amino* group of the other with the elimination of a molecule of water,[4] *e. g.*, *dipeptides, tripeptides, tetrapeptides, pentapeptides.*

CLASSIFICATION OF PROTEINS ADOPTED BY THE CHEMICAL AND PHYSIOLOGICAL SOCIETIES OF ENGLAND.

I. Simple Proteins.

1. Protamines, *e. g.*, *salmine, clupeine.*
2. Histones, *e. g.*, *globin, scombrone.*
3. Albumins, *e. g.*, *ovalbumin, serum albumin, vegetable albumins.*
4. Globulins, *e. g.*, *serum globulin, ovoglobulin, vegetable globulins.*
5. Glutelins, *e. g.*, *glutenin.*
6. Alcohol-soluble proteins, *e. g.*, *zein, gliadin.*
7. Scleroproteins, *e. g.*, *elastin, keratin.*
8. Phosphoproteins, *e. g.*, *caseinogen, vitellin.*

II. Conjugated Proteins.

1. Glucoproteins, *e. g.*, *mucins, mucoids.*
2. Nucleoproteins, *e. g.*, *nucleohistone, cytoglobulin.*
3. Chromoproteins, *e. g.*, *hæmoglobin, hæmocyanin.*

[1] The term secondary protein derivatives is used because the formation of the primary derivatives usually precedes the formation of these secondary derivatives.

[2] As thus defined, this term does not strictly cover all the protein derivatives commonly called proteoses, *e. g.*, heteroproteose and dysproteose.

[3] In this group the kyrines may be included. For the present it is believed that it will be helpful to retain this term as defined, reserving the expression *peptide* for the simpler compounds of definite structure, such as dipeptides, etc.

[4] The peptones are undoubtedly peptides or mixtures of peptides, the latter term being at present used to designate those of definite structure.

III. PRODUCTS OF PROTEIN HYDROLYSIS.

1. Infraproteins, *e. g.*, *acid infraprotein* (*acid albuminate*), *alkali infraprotein* (*alkali albuminate*).
2. Proteoses, *e. g.*, *protoproteose*, *heteroproteose*, *deuteroproteose*.
3. Peptones, *e. g.*, *amphopeptone*, *antipeptone*.
4. Polypeptides, *e. g.*, *dipeptides*, *tripeptides*, *tetrapeptides*.

CONSIDERATIONS OF THE VARIOUS CLASSES OF PROTEINS.

SIMPLE PROTEINS.

The simple proteins are true protein substances which, upon hydrolysis, yield *only* α-amino acids or their derivatives. "Although no means are at present available whereby the chemical individuality of any protein can be established, a number of simple proteins have been isolated from animal and vegetable tissues which have been so well characterized by constancy of ultimate composition and uniformity of physical properties that they may be treated as chemical individuals until further knowledge makes it possible to characterize them more definitely." Under simple proteins we may class albumins, globulins, glutelins, prolamins, albuminoids, histones and protamines.

ALBUMINS.

Albumins constitute the first class of simple proteins and may be defined as simple proteins which are coagulable by heat and soluble in pure (salt-free) water. Those of animal origin are not precipitated upon saturating their *neutral* solutions at 30° C. with sodium chloride or magnesium sulphate, but if a saturated solution of this character be acidified with acetic acid the albumin precipitates. All albumins of *animal* origin may be precipitated by saturating their solutions with ammonium sulphate.[1] They may be thrown out of solution by the addition of a sufficient quantity of a mineral acid, whereas a weak acidity produces a slight precipitate which dissolves upon agitating the solution. Metallic salts also possess the property of precipitating albumins, some of the precipitates being soluble in excess of the reagent, whereas others are insoluble in such an excess. Of those pro-

[1] In this connection, Osborne's observation that there are certain *vegetable* albumins which are precipitated by saturating their solutions with sodium chloride or magnesium sulphate or by half-saturating with ammonium sulphate, is of interest.

teins which occur native the albumins contain the highest percentage of sulphur, ranging from 1.6 to 2.5 per cent. Some albumins have been obtained in crystalline form, notably egg albumin, serum albumin, and lactalbumin, but the fact that they may be obtained in crystalline form does not necessarily prove them to be chemical individuals.

GENERAL COLOR REACTIONS OF PROTEINS.

These color reactions are due to a reaction between some one or more of the constituent radicals or groups of the complex protein molecule and the chemical reagent or reagents used in any given test. Not all proteins contain the same groups and for this reason the various color tests will yield reactions varying in intensity of color according to the nature of the groups contained in the particular protein under examination. Various substances not proteins respond to certain of these color reactions, and it is therefore essential to submit the material under examination to several tests before concluding definitely regarding its nature.

TECHNIQUE OF THE COLOR REACTIONS.

1. **Millon's Reaction.**—To 5 c.c. of a dilute solution of egg albumin in a test-tube add a few drops of Millon's reagent. A white precipitate forms which turns red when heated. This test is a particularly satisfactory one for use on *solid* proteins, in which case the reagent is added directly to the solid substance and heat applied, which causes the substance to assume a red color. Such proteins as are not precipitated by mineral acids, for example certain of the proteoses and peptones, yield a *red solution* instead of a red precipitate.

The reaction is due to the presence of the *hydroxy-phenyl group*, $-C_6H_4OH$, in the protein molecule and certain non-proteins such as tyrosine, phenol (carbolic acid) and thymol also respond to the reaction. Inasmuch as the tyrosine grouping is the only hydroxy-phenyl grouping which has definitely been proven to be present in the protein molecule it is evident that protein substances respond to Millon's reaction because of the presence of this tyrosine complex. The test is not a very satisfactory one for use in solutions containing inorganic salts in large amount, since the mercury of the Millon's reagent[1] is thus precipitated and the reagent rendered inert. This

[1] Millon's reagent consists of mercury dissolved in nitric acid containing some nitrous acid. It is prepared by digesting one part (by weight) of mercury with two parts (by weight) of HNO_3 (sp. gr. 1.42) and diluting the resulting solution with two volumes of water.

reagent is therefore never used for the detection of protein material in the urine.

2. **Xanthoproteic Reaction.**—To 2–3 c.c. of egg albumin solution in a test-tube add concentrated nitric acid. A white precipitate forms, which upon heating turns yellow and finally dissolves, imparting to the solution a yellow color. *Cool the solution* and carefully add ammonium hydroxide, potassium hydroxide, or sodium hydroxide *in excess*. Note that the yellow color deepens into an orange. This reaction is due to the presence in the protein molecule of the *phenyl group*, with which the nitric acid forms certain nitro modifications. The particular complexes of the protein molecule which are of especial importance in this connection are those of tyrosine, phenylalanine, and tryptophane. The test is not a satisfactory one for use in urinary examination because of the color of the end-reaction.

3. **Adamkiewicz Reaction.**—Thoroughly mix 1 volume of concentrated sulphuric acid and 2 volumes of acetic acid in a test-tube, add a few drops of egg albumin solution and heat gently. A reddish-violet color is produced. Gelatin does not respond to this test. This reaction shows the presence of the *tryptophane group* (see next experiment). The test depends upon the presence of glyoxylic acid, CHO.COOH+H_2O or $CH(OH)_2COOH$, in the reagents. This is shown by the failure to secure a positive reaction when acetic acid free from glyoxylic acid is used.

Rosenheim has recently advanced the view that the reaction may be due to the presence of oxidizing agents such as nitrous acid and ferric salts in the sulphuric acid.

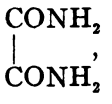
4. **Hopkins-Cole Reaction.**[1]—Place 1–2 c.c. of egg albumin solution and 3 c.c. of glyoxylic acid, CHO.COOH+H_2O or $CH(OH)_2$-COOH, solution (Hopkins-Cole reagent[2]) in a test-tube and mix thoroughly. In a second tube place 5 c.c. of concentrated sulphuric acid. Incline the tube containing the sulphuric acid and by means of a pipette allow the albumin-glyoxylic acid solution to flow *carefully* down the side. When stratified in this manner a reddish-violet color forms at the zone of contact of the two fluids. This color is due to the presence of the *tryptophane group*. Gelatin does not respond to this test. For formula for tryptophane see page 72.

[1] Hopkins and Cole: *Journal of Physiology*, XXVII, p. 418, 1902.

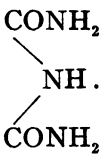
[2] Hopkins-Cole reagent is prepared as follows: To one liter of a saturated solution of oxalic acid add 60 grams of sodium amalgam and allow the mixture to stand until the evolution of gas ceases. Filter and dilute with 2–3 volumes of water.

Benedict[1] has recently suggested a new reagent for use in carrying out the Hopkins-Cole reaction.[2]

5. **Biuret Test.**—To 2–3 c.c. of egg albumin solution in a test-tube add an equal volume of concentrated potassium hydroxide solution, mix thoroughly, and add slowly a very dilute (2–5 drops in a test-tube of water) cupric sulphate solution until a purplish-violet or pinkish-violet color is produced. The depth of the color depends upon the nature of the protein; proteoses, and peptones giving a decided pink, while the color produced with gelatin is not far removed from a blue. This reaction is given by those substances which contain *two amino groups* in their molecule, these groups either being joined directly together or through a single atom of nitrogen or carbon. The amino groups mentioned must either be two $CONH_2$ groups or one $CONH_2$ group and one $CSNH_2$, $C(NH)NH_2$ or CH_2NH_2 group. It follows from this fact that substances which are non-protein in character but which contain the necessary groups will respond to the biuret test. As examples of such substances may be cited *oxamide*,



and *biuret*,



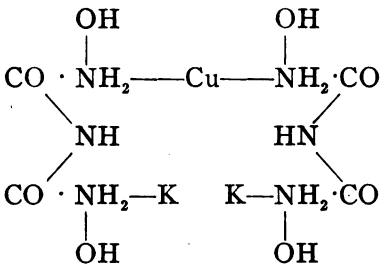
The test derives its name from the fact that this latter substance which is formed on heating urea to 180° C. (see page 262), will respond to the test. Protein material responds positively since there are two $CONH_2$ groups in the protein molecule.

According to Schiff the end-reaction of the biuret test is dependent upon the formation of a copper-potassium-biuret compound (cupri-

[1] Benedict: *Journal of Biological Chemistry*, VI, p. 51, 1909.

[2] Benedict's modified Hopkins-Cole reagent is prepared as follows: Ten grams of powdered magnesium are placed in a large Erlenmeyer flask and shaken up with enough distilled water to liberally cover the magnesium. Two hundred and fifty c.c. of a cold, saturated solution of oxalic acid is now added slowly. The reaction proceeds very rapidly and with the liberation of much heat, so that the flask should be cooled under running water during the addition of the acid. The contents of the flask are shaken after the addition of the last portion of the acid and then poured upon a filter, to remove the insoluble magnesium oxalate. A little wash water is poured through the filter, the filtrate acidified with acetic acid to prevent the partial precipitation of the magnesium on long standing, and made up to a liter with distilled water. This solution contains only the magnesium salt of glyoxylic acid.

potassium biuret or biuret potassium cupric hydroxide). This substance was obtained by Schiff in the form of long red needles. It has the following formula:



Gies[1] has recently devised a reagent for use in the biuret test. This reagent consists of 10 per cent KOH solution, to which enough 3 per cent $CuSO_4$ solution has been added to impart a slight though distinct blue color to the clear liquid. The $CuSO_4$ should be added drop by drop with thorough shaking after each addition. This reagent is of material assistance in performing the biuret test.

6. **Posner's Modification of the Biuret Test.**—This test is particularly satisfactory for use on *dilute* protein solutions, and is carried out as follows: To some dilute egg albumin in a test-tube add one-half its volume of potassium hydroxide solution. Now hold the tube in an inclined position and allow some very dilute cupric sulphate solution, made as suggested on page 90 (5), to flow down the side, being especially careful to prevent the fluids from mixing. At the juncture of the two solutions the typical end-reaction of the biuret test should appear as a colored zone (see Biuret Test, page 90).

7. **Liebermann's Reaction.**—Add about 10 drops of *concentrated* egg albumin solution (or a little dry egg albumin) to about 5 c.c. of concentrated HCl in a test-tube. Boil the mixture until a pinkish-violet color results. This color was originally supposed to indicate the presence of a carbohydrate group in the protein molecule, the furfurol formed through the action of the acid upon the protein reacting with the *hydroxy-phenyl group* of the protein producing the pinkish-violet color. It is now considered *uncertain* whether the carbohydrate group enters into the reaction. Cole has called attention to the fact that a *blue* color results if protein material which has been boiled with alcohol and subsequently *washed with ether* be used in making the test. He believes the blue color to be due to an interaction between the

[1] Gies: Proceedings of Society of Biological Chemists, *Journal of Biological Chemistry*, VII, p 60, 1910.

glyoxylic acid, which was present as an impurity in the ether used in washing the protein, and the *tryptophane group* of the protein molecule which was split off through the action of the acid.

8. **Acree-Rosenheim Formaldehyde Reaction.**—Add a few drops of a dilute (1 : 5000) solution of formaldehyde to 2–3 c.c. of egg albumin solution in a test-tube. Mix thoroughly and after 2–3 minutes carefully introduce a little concentrated sulphuric acid into the tube in such a manner that the two solutions do not mix. A violet zone will be observed at the point of juncture of the two solutions especially if the mixture is slightly agitated. This color probably results through the union of the protein and the formaldehyde. If the sulphuric acid is added to the protein *before* the formaldehyde is added the typical end-reaction is not obtained. So far as is known this is a specific test for proteins. The reaction cannot be applied satisfactorily with concentrated formaldehyde.

Rosenheim claims the reaction is due to the presence of oxidizing material in the sulphuric acid and that when pure sulphuric acid is used no reaction is obtained. He advises the use of a slight amount of an oxidizing agent, *e. g.*, ferric chloride or potassium nitrate (0.005 gram per 100 c.c. of sulphuric acid) in order to facilitate the reaction. Rosenheim further states that proteins respond to the formaldehyde reaction because of the presence of the *tryptophane group*, a statement which Acree does not accept as proven.

9. **Bardach's Reaction.**[1]—This is one of the most recent tests which have been described for the detection of protein material. The test depends upon the property possessed by protein substances of preventing the formation of typical iodoform crystals through the interaction of an alkaline acetone solution with iodopotassium iodide. Instead of the typical hexagonal plates or stellar formations of iodoform there are produced, under the conditions of the test, *fine yellow needles* which are apparently some iodine compound other than iodoform. The technique of the test is as follows: Place about 5 c.c. of the protein solution[2] under examination in a test-tube, add 2–3 drops of a 0.5 per cent solution of acetone and sufficient Lugol's solution[3] to supply a moderate excess of iodine and produce a red-brown coloration. (The amount of Lugol's solution necessary will depend upon the content of protein, sugar, and other iodine-reacting substances in the solu-

[1] Bardach: *Zeitschrift für Physiologische Chemie*, 1908, LIV, p. 355; also Seaman and Gies: *Proceedings of the Society for Experimental Biology and Medicine*, 1908, V, p. 125.

[2] The solution should not contain more than 5 per cent of protein material.

[3] Dissolve 4 grams of iodine and 6 grams of potassium iodide in 100 c.c. of distilled water.

tion under examination and may vary from one drop to several cubic centimeters.) Add an excess (ordinarily about 3 c.c.) of concentrated ammonium hydroxide and thoroughly mix the solution. Place the tube in the test-tube rack, examine the contents at intervals of five minutes, and when it is evident that crystals have formed, place a drop of the mixture upon a microscopic slide, put a coverglass in position, and examine the mixture under the microscope. The formation of *canary yellow crystals* indicates the presence of protein material in the solution examined. The crystals are ordinarily needle-like in appearance and show a tendency to assume rosette or bundle-like formations, but under certain conditions they may show knobbed (nail-like) and branching variations.

If a moderate excess of iodine is used in making the test, a black precipitate of iodonitro compounds is at once formed upon the addition of the ammonium hydroxide, and yellow needles are subsequently deposited upon it. In case just the proper amount of iodine is used, the solution soon assumes a yellow color and the black precipitate formed upon the addition of the ammonium hydroxide is gradually transformed more or less completely into the yellow crystals. In either case the needles ordinarily form within an hour, and frequently in a much shorter time. If too great an excess of iodine is employed the heavy black precipitate may obscure or even prevent the reaction. The presence of insufficient iodine or excess protein may likewise prevent the reaction. In tests in which a concentrated protein solution and an excess of iodine are used, the addition of ammonium hydroxide immediately produces a grayish-green precipitate. In such instances, if the proportions are favorable, and the mixture be stirred with a glass rod for a few minutes, the precipitate is gradually transformed into the crystals before mentioned.

It is probable that all soluble proteins will respond to Bardach's reaction, but the relative delicacy of the reaction as well as the value of the test as compared with other protein tests remain to be determined. The only disturbing factor noted thus far is the presence of earthy phosphates in the solution under examination.

PRECIPITATION REACTIONS AND OTHER PROTEIN TESTS.

There are three forms in which proteins may be precipitated, *i. e.*, *unaltered*, as an *albuminate*, and as an *insoluble salt*. An instance of the precipitation in a *native* or *unaltered* condition is seen in the so-called *salting-out* experiments. Various salts, notably $(NH_4)_2SO_4$,

$ZnSO_4$, $MgSO_4$, Na_2SO_4 and NaCl possess the power when added in *solid form* to certain definite protein solutions, of rendering the menstruum incapable of holding the protein in solution, thereby causing the protein to be precipitated or *salted-out* to use the common term. Mineral acids and alcohol also precipitate proteins unaltered. Proteins are precipitated as *albuminates* when treated with certain metallic salts, and precipitated as *insoluble salts* when weak organic acids such as certain of the alkaloidal reagents are added to their solutions.

It is generally stated that globulins are precipitated from their solutions upon *half* saturation with ammonium sulphate and that albumins are precipitated upon *complete* saturation by this salt. Comparatively few exceptions were found to this rule until proteins of *vegetable* origin came to be more extensively studied. These studies, furthered especially by Osborne and associates, have demonstrated very clearly that the characterization of a globulin as a protein which is precipitated by *half* saturation with ammonium sulphate, can no longer hold. Certain vegetable globulins have been isolated which are not precipitated by this salt until a concentration is reached *greater than that secured by half-saturation.* As an example of an albumin which does not conform to the definition of an albumin as regards its precipitation by ammonium sulphate, may be mentioned the *leucosin* of the wheat germ which is precipitated from its solution upon *half*-saturation with ammonium sulphate. The limits of precipitation by ammonium sulphate, therefore, do not furnish a sufficiently accurate basis for the differentiation of globulins from albumins. It has further been determined that a given protein which is precipitable by ammonium sulphate cannot be "salted-out" by the same concentration of the salt under all conditions.

Experiments.

1. **Influence of Concentrated Mineral Acids, Alkalis and Organic Acids.**—Prepare five test-tubes each containing 5 c.c. of concentrated egg albumin solution. To the first add concentrated H_2SO_4, drop by drop, until an excess of the acid has been added. Note any changes which may occur in the solution. Allow the tube to stand for 24 hours and at the end of that period observe any alteration which may have taken place. Heat the tube and note any further change which may occur. Repeat the experiment in the four remaining tubes with concentrated hydrochloric acid, concentrated nitric acid, concentrated potassium hydroxide and acetic acid. How do

strong mineral acids, strong alkalis, and strong organic acids differ in their action toward protein solutions?

2. **Precipitation by Metallic Salts.**—Prepare four tubes each containing 2–3 c.c. of dilute egg albumin solution. To the first add *mercuric chloride, drop by drop,* until an excess of the reagent has been added, noting any changes which may occur. Repeat the experiment with *plumbic acetate, argentic nitrate, cupric sulphate, ferric chloride,* and *barium chloride.*

Egg albumin is used as an antidote for lead or mercury poisoning. Why?

3. **Precipitation by Alkaloidal Reagents.**—Prepare six tubes each containing 2–3 c.c. of dilute egg albumin solution. To the first add *picric acid* drop by drop until an excess of the reagent has been added, noting any changes which may occur. Repeat the experiment with *trichloracetic acid, tannic acid, phosphotungstic acid, phospho-molybdic acid,* and *potassio-mercuric iodide.* Acidify with hydrochloric acid before testing with the three last reagents.

4. **Heller's Ring Test.**—Place 5 c.c. of concentrated nitric acid in a test-tube, incline the tube, and by means of a pipette allow the dilute albumin solution to flow *slowly* down the side. The liquids should stratify with the formation of a white zone of precipitated albumin at the point of juncture. This is a very delicate test and is further discussed on p. 308.

An apparatus called the *albumoscope* or *horismascope* has been devised for use in the tests of this character and has met with considerable favor. The method of using the albumoscope is described below.

Use of the Albumoscope.—This instrument is intended to facilitate the making of "ring" tests such as Heller's and Roberts'. In making a test about 5 c.c. of the solution under examination is first introduced into the apparatus through the larger arm and the reagent used in the particular test is then introduced through the capillary arm and allowed to flow down underneath the solution under examination. If a reasonable amount of care is taken there is no possibility of mixing the two solutions and a definitely defined white "ring" is easily obtained at the zone of contact.

5. **Roberts' Ring Test.**—Place 5 c.c. of Roberts' reagent[1] in a test-tube, incline the tube, and by means of a pipette allow the albumin solution to flow *slowly* down the side. The liquids should stratify with the formation of a *white* zone of precipitated albumin at

[1] Roberts' reagent is composed of 1 volume of concentrated HNO_3 and 5 volumes of a saturated solution of $MgSO_4$.

the point of juncture. This test is a modification of Heller's ring test and is rather more satisfactory. The albumoscope may also be used in making this test. (See page 95.)

6. **Spiegler's Ring Test.**—Place 5 c.c. of Spiegler's reagent[1] in a test-tube, incline the tube, and by means of a pipette allow 5 c.c. of albumin solution, acidified with acetic acid, to flow slowly down the side. A white zone will form at the point of contact. This is an exceedingly delicate test, in fact too delicate for ordinary clinical purposes, since it serves to detect albumin when present in the merest trace (1:250,000). This test is further discussed on page 310.

7. **Jolles' Reaction.**—Shake 5 c.c. of albumin solution with 1 c.c. of 30 per cent acetic acid and 4 c.c. of Jolles' reagent[2] in a test-tube. A *white* precipitate of albumin should form. Care should be taken to use the correct amount of acetic acid. For further discussion of the test see page 310.

8. **Tanret's Test.**—To 5 c.c. of albumin solution in a test-tube add Tanret's reagent,[3] drop by drop, until a turbidity or precipitate forms. This is an exceedingly delicate test. Sometimes the albumin solution is stratified upon the reagent as in Heller's or Roberts' ring tests. In urine examination it is claimed by Repiton that the presence of urates lowers the delicacy of the test. Tanret has, however, very recently made a statement to the effect that the removal of urates is not necessary inasmuch as the urate precipitate will disappear on warming and the albumin precipitate will not. He says, however, that mucin interferes with the delicacy of his test and should be removed by acidification with acetic acid and filtration before testing for albumin.

9. **Sodium Chloride and Acetic Acid Test.**—Mix 2 volumes of albumin solution and 1 volume of a saturated solution of sodium chloride in a test-tube, acidify with acetic acid, and heat to boiling. The production of a cloudiness or the formation of a precipitate indicates the presence of albumin.

[1] Spiegler's reagent has the following composition:

Tartaric acid	20 grams.
Mercuric chloride	40 grams.
Glycerol	100 grams.
Distilled water	1000 grams.

[2] Jolles' reagent has the following composition:

Succinic acid	40 grams.
Mercuric chloride	20 grams.
Sodium chloride	20 grams.
Distilled water	1000 grams.

[3] Tanret's reagent is prepared as follows: Dissolve 1.35 gram of mercuric chloride in 25 c.c. of water, add to this solution 3.32 grams of potassium iodide dissolved in 25 c.c. of water, then make the total solution up to 60 c.c. with water and add 20 c.c. of glacial acetic acid to the combined solutions.

10. **Potassium Iodide Test.**—Stratify a dilute albumin solution upon a solution of potassium iodide made slightly acid with acetic acid. In the presence of 0.01–0.02 per cent of albumin a white ring forms immediately. If the test be allowed to stand two minutes after the stratification it will serve to detect 0.005 per cent of albumin.

11. **Acetic Acid and Potassium Ferrocyanide Test.**—To 5 c.c. of dilute egg albumin solution in a test-tube add 5–10 drops of acetic acid. Mix well, and add potassium ferrocyanide, *drop by drop*, until a precipitate forms. This test is very delicate.

Schmiedl claims that a precipitate of $Fe(Cn)_6K_2Zn$ or $Fe(Cn)_6Zn_2$, is formed when solutions containing zinc are subjected to this test, and that this precipitate resembles the precipitate secured with protein solutions. In the case of human urine a reaction was obtained when 0.000022 gram of zinc per cubic centimeter was present. Schmiedl further found that the urine collected from rabbits housed in zinc-lined cages possessed a zinc content which was sufficient to yield a ready response to the test. Zinc is the only interfering substance so far reported.

12. **Salting-out Experiments.**—(*a*) To 25 c.c. of egg albumin solution in a small beaker add *solid* ammonium sulphate to the point of saturation, keeping the temperature of the solution below 40° C. Filter, test the precipitate by Millon's reaction and the filtrate by the biuret test. What are your conclusions? (*b*) Repeat the above experiment making the saturation with *solid* sodium chloride. How does this result differ from the result of the saturation with ammonium sulphate? Add 2–3 drops of acetic acid. What occurs? All proteins *except peptones* are precipitated by saturating their solutions with ammonium sulphate. *Globulins* are the only proteins precipitated by saturating with sodium chloride (see Globulins, page 100), unless the saturated solution is subsequently acidified, in which event all proteins *except peptones* are precipitated.

Soaps may be salted-out in a similar manner (see p. 134).

13. **Coagulation or Boiling Test.**—Heat 25 c.c. of dilute egg albumin solution to the boiling-point in a small evaporating dish. The albumin coagulates. Complete coagulation may be obtained by acidifying the solution with 3–5 drops of acetic acid[1] *at the boiling-point.* Test the coagulum by Millon's reaction. The acid is added to neutralize any possible alkalinity of the solution, and to dissolve any substances which are not albumin (see further discussion on page 311).

[1] Nitric acid is often used in place of acetic acid in this test. In case nitric acid is used, ordinarily 1–2 drops is sufficient.

14. **Coagulation Temperature.**—Prepare 4 test-tubes each containing 5 c.c. of *neutral* egg albumin solution. To the first add 1 drop of 0.2 per cent hydrochloric acid, to the second add 1 drop of 0.5 per cent sodium carbonate solution, to the third add 1 drop of 10 per cent sodium chloride solution and leave the fourth neutral in reaction. Partly fill a beaker of medium size with water and place it within a second larger beaker which also contains water, the two vessels being separated by pieces of cork. Fasten the four test-tubes compactly together by means of a rubber band, lower them into the water of the inner beaker and suspend them, by means of a clamp attached to one of the tubes, in such a manner that the albumin solutions shall be midway between the upper and lower surfaces of the water. In one of the tubes place a thermometer with its bulb entirely beneath the surface of the albumin solution (Fig. 32). Gently heat the water in the beakers, noting carefully any changes which may occur in the albumin solutions and record the exact temperature at which these changes occur. The first appearance of an *opacity* in an albumin solution indicates the commencement of coagulation and the temperature at which this occurs should be recorded as the *coagulation temperature* for that particular albumin solution.

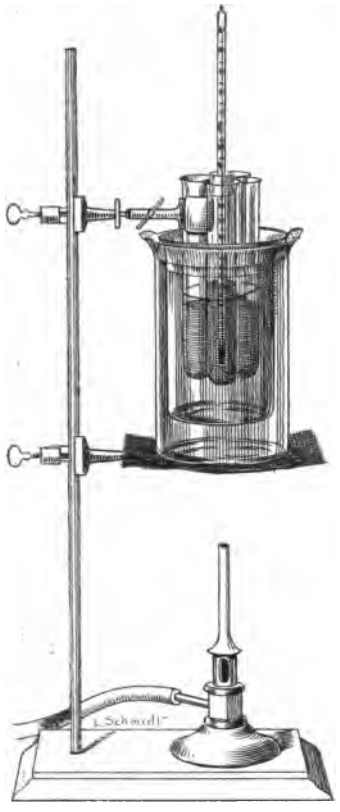


FIG. 32.—COAGULATION TEMPERATURE APPARATUS.

What is the order in which the four solutions coagulate?

Repeat the experiment, adding to the first tube 1 drop of acetic acid, to the second 1 drop of concentrated potassium hydroxide solution, to the third 2 drops of a 10 per cent sodium chloride solution and leave the fourth neutral as before.

What is the order of coagulation here? Why?

15. **Precipitation by Alcohol.**—Prepare 3 test-tubes each containing about 10 c. c. of 95 per cent alcohol. To the first add one drop of 0.2 per cent hydrochloric acid, to the second one drop of potassium

hydroxide solution and leave the third neutral in reaction. Add to each tube a few drops of egg albumin solution and note the results. What do you conclude from this experiment? Alcohol precipitates proteins unaltered, but if allowed to remain under alcohol the protein is transformed. The "fixing" of tissues for histological examination by means of alcohol is an illustration of the application of this transformation produced by alcohol. It apparently is a process of dehydrolysis.

16. **Preparation of Powdered Egg Albumin.**—This may be prepared as follows: Ordinary egg-white finely divided by means of scissors or a beater is treated with four volumes of water and filtered. The filtrate is evaporated on a water-bath at about 50° C. and the residue powdered in a mortar.

17. **Tests on Powdered Egg Albumin.**—With powdered albumin prepared as described above (by yourself or furnished by the instructor), try the following tests:

(*a*) *Solubility.*

(*b*) *Millon's Reaction.*

(*c*) *Hopkins-Cole Reaction.*—When used to detect the presence of protein in solid form this reaction should be conducted as follows: Place 5 c.c. of concentrated sulphuric acid in a test-tube and add carefully, by means of a pipette, 3–5 c.c. of Hopkins-Cole reagent. Introduce a small amount of the solid substance to be tested, agitate the tube slightly, and note that the suspended pieces assume a reddish-violet color, which is the characteristic end-reaction of the Hopkins-Cole test; later the solution will also assume the reddish-violet color.

(*d*) *Composition Test.*—Heat some of the powder in a test-tube in which is suspended a strip of moistened red litmus paper and across the mouth of which is placed a piece of filter paper moistened with plumbic aceate solution. As the powder is heated it chars, indicating the presence of *carbon;* the fumes of ammonia are evolved, turning the red litmus paper blue and indicating the presence of *nitrogen* and *hydrogen;* the plumbic acetate paper is blackened, indicating the presence of sulphur, and the deposition of moisture on the side of the tube indicates the presence of *hydrogen.*

(*e*) Immerse a dry test-tube containing a little powdered egg albumin in boiling water for a few moments. Remove and test the solubility of the albumin according to the directions given under (*a*) above. It is still soluble. Why has it not been coagulated? Repeat the above experiments with powdered serum albumin and see how the results compare with those just obtained.

SULPHUR IN PROTEIN.

Sulphur is believed to be present in two different forms in the protein molecule. The first form, which is present in greatest amount, is that loosely combined with carbon and hydrogen. Sulphur in this form is variously termed *unoxidized*, *loosely combined*, *mercaptan*, and *lead-blackening* sulphur. The second form is combined in a more stable manner with carbon and oxygen and is known as *oxidized* or *acid* sulphur. The protamines are the only class of sulphur-free proteins.

TESTS FOR SULPHUR.

1. **Test for Loosely Combined Sulphur.**—To equal volumes of KOH and egg albumin solutions in a test-tube add 1–2 drops of plumbic acetate solution and boil the mixture. Loosely combined sulphur is indicated by a darkening of the solution, the color deepening into a black if sufficient sulphur is present. Add hydrochloric acid and note the characteristic odor evolved from the solution. Write the reactions for this test.

2. **Test for Total Sulphur (Loosely Combined and Oxidized).**—Place the substance to be examined (powdered egg albumin) in a small procelain crucible, add a suitable amount of solid fusion mixture (potassium hydroxide and potassium nitrate mixed in the proportion 5 : 1) and heat carefully until a colorless mixture results. (Sodium peroxide may be used in place of this fusion mixture if desired.) Cool, dissolve the cake in a little warm water and filter. Acidify the filtrate with hydrochloric acid, heat it to the boiling-point and add a small amount of barium chloride solution. A white precipitate forms if sulphur is present. What is this precipitate?

GLOBULINS.

Globulins are simple proteins especially predominant in the vegetable kingdom. They are closely related to the albumins and in common with them give all the ordinary protein tests. Globulins differ from the albumins in being insoluble in pure (salt-free) water. They are, however, soluble in neutral solutions of salts of strong bases with strong acids. Most globulins are precipitated from their solutions by saturation with solid sodium chloride or magnesium sulphate. As a class they are much less stable than the albumins, a fact shown by the increasing difficulty with which a globulin dissolves during the course of successive reprecipitations.

We have used an albumin of animal origin (egg albumin) for all the protein tests thus far, whereas the globulin to be studied will be prepared from a vegetable source. There being no essential difference between animal and vegetable proteins, the vegetable globulin we shall study may be taken as a true type of all globulins, both animal and vegetable.

Experiments on Globulin.

Preparation of the Globulin.—Extract 20–30 grams (a handful) of crushed hemp seed with a 5 per cent solution of sodium chloride for one-half hour at 60° C. Filter while hot through a paper moistened with 5 per cent sodium chloride solution. Place the filtrate in the water-bath at 60° C. and allow it to stand for 24 hours in order that the globulin may crystallize slowly. In case the filtrate is *cloudy* it

Fig. 33.—Edestin.

should be warmed to 60° C. in order to produce a *clear* solution. The globulin is soluble in *hot* 5 per cent sodium chloride solution and is thus extracted from the hemp seed, but upon cooling this solution much of the globulin separates in crystalline form. This particular globulin is called *edestin*. It crystallizes in several different forms, chiefly octahedra (see Fig. 33, above). (The crystalline form of *excelsin*, a protein obtained from the Brazil nut, is shown in Fig. 34, page 103. This vegetable protein crystallizes in the form of hexagonal plates.) Filter off the edestin and make the following tests on the crystalline body and on the filtrate which still contains some of the extracted globulin.

TESTS ON CRYSTALLIZED EDESTIN.—(1) *Microscopical examination* (see Fig. 33, p. 101).

(2) *Solubility.*—Try the solubility in the ordinary solvents (see page 22). Keep these solubilities in mind for comparison with those of edestan, to be made later (see page 107).

(3) *Millon's Reaction.*

(4) *Coagulation Test.*—Place a small amount of the globulin in a test-tube, add a little water and boil. Now add dilute hydrochloric acid and note that the protein no longer dissolves. It has been coagulated.

(5) Dissolve the remainder of the edestin in 0.2 per cent hydrochloric acid and preserve this acid solution for use in the experiments on proteans (see page 106).

TESTS ON EDESTIN FILTRATE.—(1) *Influence of Protein Precipitants.*—Try a few protein precipitants such as *nitric acid, tannic acid, picric acid,* and *mercuric chloride.*

(2) *Biuret Test.*

(3) *Coagulation Test.*—Boil some of the filtrate in a test-tube. What happens?

(4) *Saturation with Sodium Chloride.*—Saturate some of the filtrate with *solid* sodium chloride. How does this result differ from that obtained upon saturating egg albumin solution with *solid* sodium chloride?

(5) *Precipitation by Dilution.*—Dilute some of the filtrate with 10–15 volumes of water. Why does the globulin precipitate?

Glutelins.

It has been repeatedly shown, particularly by Osborne, that after extracting the seeds of cereals with water, neutral salt solution, and strong alcohol, there still remains a residue which contains protein material which may be extracted by very dilute acid or alkali. These proteins which are insoluble in all neutral solvents, but readily soluble in very dilute acids and alkalis are called *glutelins.* The only member of the group which has yet received a name, is the *glutenin* of wheat, a protein which constitutes nearly 50 per cent of the gluten. It is not definitely known whether glutelins occur as constituents of all seeds.

Prolamins (Alcohol-soluble Proteins).

The term prolamin has been proposed by Osborne, for the group of proteins formerly termed "alcohol-soluble proteins." The name

is very appropriate inasmuch as these proteins yield, upon hydrolysis, especially large amounts of *proline* and *ammonia*. The prolamins are simple proteins which are insoluble in water, *absolute* alcohol and other neutral solvents, but are soluble in 70 to 80 per cent alcohol and in dilute acids and alkalis. They occur widely distributed, particularly in the vegetable kingdom. The only prolamins yet described are the *zein* of maize, the *hordein* of barley, the *gliadin* of wheat and rye, and the *bynin* of malt. They yield relatively large amounts of glutamic

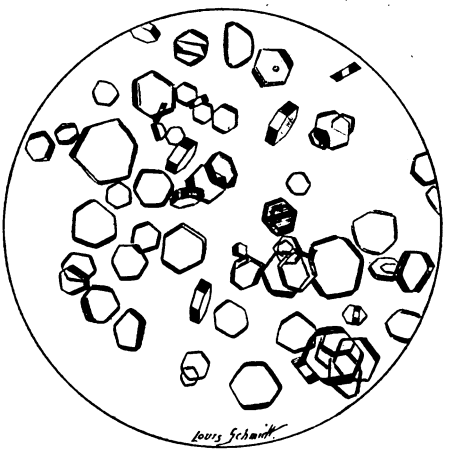


FIG. 34.—EXCELSIN, THE PROTEIN OF THE BRAZIL NUT.
(Drawn from crystals furnished by Dr. Thomas B. Osborne, New Haven, Conn.)

acid on hydrolysis but *no lysin*. The largest percentage of glutamic acid (41.32 per cent) ever obtained as a decomposition product of a protein substance has very recently been obtained by Kleinschmitt from the hydrolysis of the prolamin hordein.[1] This yield of glutamic acid is also the largest amount of any single decomposition product yet obtained from any protein *except protamines*.

Albuminoids. (Scleroproteins.)

The albuminoids yield similar hydrolytic products to those obtained from the other simple proteins already considered, thus indicating that they possess essentially the same chemical structure. They differ from all other proteins, whether simple, conjugated, or derived, in that they are insoluble in all neutral solvents. The albuminoids include "the principal organic constituents of the skeletal structure of animals

[1] Up to this time the yield of 37.33 per cent obtained by Osborne and Harris from gliadin, was the maximum yield.

as well as their external covering and its appendages. Some of the principal albuminoids are *keratin*, *elastin*, *collagen*, *reticulin*, *spongin*, and *fibroin*. Gelatin cannot be classed as an albuminoid although it is a transformation product of collagen. The various albuminoids differ from each other in certain fundamental characteristics which will be considered in detail under Epithelial and Connective Tissue (see Chapter XIV, p. 223).

CONJUGATED PROTEINS.

Conjugated proteins consist of a protein molecule united to some other molecule or molecules otherwise than as a salt. We have *glycoproteins*, *nucleoproteins*, *hæmoglobins* (chromoproteins), *phosphoproteins* and *lecithoproteins* as the five classes of conjugated proteins.

Glycoproteins may be considered as compounds of the protein molecule with a substance or substances containing a carbohydrate group other than a nucleic acid. The *glycoproteins* yield, upon decomposition, protein and carbohydrate derivatives, notably glycosamine, $CH_2OH.(CHOH)_3.CH(NH_2).CHO$, and galactosamine, $OHCH_2.(CHOH)_3.CH(NH_2).CHO$. The principal glycoproteins are *mucoids*, *mucins*, and *chondroproteins*. By the term *mucoid* we may designate those glycoproteins which occur in tissues, such as *tendomucoid* from tendinous tissue and *osseomucoid* from bone. The elementary composition of these typical mucoids is as follows:

	N.	S.	C.	H.	O	
Tendomucoid	11.75	2.33	48.76	6.53	30.60	(Chittenden and Gies)
Osseomucoid	12.22	2.32	47.43	6.63	31.40	

The term *mucins* may be said to include those forms of glycoproteins which occur in the secretions and fluids of the body. Chondroproteins are so named because *chondromucoid*, the principal member of the group, is derived from cartilage (chondrigen). *Amyloid*, which appears pathologically in the spleen, liver, and kidneys, is also a chondroprotein.

The *nucleoproteins* occur principally in animal and vegetable cells, and following the destruction of these cells they are found in the fluids of the body. These proteins are discharged into the tissue fluids by the activity or disintegration of cells. Combined with the simple protein in the neucleoprotein molecule we find *nucleic acid*, a body which contains phosphorus and which yields *purine bases* and *pyrimidine bases* (*thymine*, *cytosine*, and *uracil*) upon decomposition. The so-called *nucleins* are formed in the gastric digestion of nucleoproteins.

Wheeler-Johnson Reaction for Uracil and Cytosine.—To about 5 c.c. of the solution under examination add bromine water until the color is permanent.[1] In case the solution contains only small quantities of cytosine or uracil, it is advisable to remove the excess of bromine by passing a stream of air through the solution. Now add an excess of an aqueous solution of barium hydroxide and note the appearance of a purple color.

Very dilute solutions do not give the test. Under these conditions the solution should be evaporated to dryness, the residue dissolved in a little bromine water and the excess of bromine removed. Then upon adding an excess of barium hydroxide a decided bluish-pink or lavender color will appear in the presence of as small an amount as 0.001 gram of uracil.

In testing solutions for cytosine, it is preferable to warm or boil the solution with bromine water, and after cooling the solution to apply the test as suggested above, being careful to have a slight excess of bromine present before adding barium hydroxide.

The *phosphoproteins* are called *nucleoalbumins* in many classifications and are grouped among the simple proteins. They are considered to be "compounds of the protein molecule and some, as yet undefined, phosphorus-containing substances other than a nucleic acid or lecithin." The percentage of phosphorus in phosphoproteins is very similar to that in nucleoproteins but they differ from this latter class of proteins in that they do not yield any purine bases upon hydrolytic cleavage. Two of the common phosphoproteins are the *caseinogen* of milk and the *ovovitellin* of the egg-yolk.

The *hæmoglobins* (chromoproteins) are compounds of the protein molecule with hæmatin or some similar substance. The principal member of the group is the hæmoglobin of the blood. Upon hydrolytic cleavage this hæmoglobin yields a protein termed *globin* and a coloring matter termed *hæmochromogen*. The latter substance contains *iron* and upon coming in contact with oxygen is oxidized to form *hæmatin*. *Hæmocyanin*, another member of the class of hæmoglobins, occurs in the blood of certain invertebrates, notably cephalopods, gasteropods, and crustacea. Hæmocyanin generally contains either *copper*, *manganese*, or *zinc* in place of the *iron* of the hæmoglobin molecule.

The *lecithoproteins* include such substances as *lecithans* and *phos-*

[1] Avoid the addition of a large excess of bromine inasmuch as this will interfere with the test.

phatides which consist of a protein molecule joined to lecithin. They have been comparatively little studied until recently, and in much of the older research they were undoubtedly considered as lecithins.

For experiments on conjugated proteins see pages 54, 191, 197, 198, 218, and 224.

DERIVED PROTEINS.

These substances are derivatives which are formed through hydrolytic changes of the original protein molecule. They may be divided into two groups, the *primary* protein derivatives and the *secondary* protein derivatives. The term secondary derivatives is made use of in this connection since the formation of the primary derivatives generally precedes the formation of these secondary derivatives. These derived proteins are obtained from native simple proteins by hydrolyses of various kinds, *e. g.*, through the action of acids, alkalis, heat, or enzymes. The particular class of derived protein desired regulates the method of treatment to which the native protein is subjected.

Primary Protein Derivatives.

The primary protein derivatives are "apparently formed through hydrolytic changes which involve only slight alterations of the protein molecule." This class includes *proteans*, *metaproteins*, and *coagulated proteins*.

PROTEANS.

Proteans are those insoluble protein substances which are produced from proteins originally soluble through the incipient action of water, enzymes, or very dilute acids. It is well known that globulins become insoluble upon repeated reprecipitation and it may possibly be found that the greater number of the proteans are transformed globulins. Osborne, however, believes that nearly all proteins may give rise to proteans. This investigator who has so very thoroughly investigated many of the vegetable proteins claims that the hydrogen ion is the active agent in the transformation. The protein produced from the transformation of *edestin* is called *edestan*, that produced from *myosin* is called *myosan*, etc. The name protean was first given to this class of proteins by Osborne in 1900 in connection with his studies of edestin.

EXPERIMENTS ON PROTEANS.

Preparation and Study of Edestan.—Prepare edestin according to the directions given on page 101. Bring the edestin into solution in

0.2 per cent hydrochloric acid and permit the acid solution to stand for about one-half hour.[1] Neutralize, with a 0.5 per cent solution of sodium carbonate, filter off the precipitate of edestan and make the following tests:

1. **Solubility.**—Try the solubility in the ordinary solvents (see page 22). Note the altered solubility of the *edestan* as compared with that of edestin (see page 101).

2. **Millon's Reaction.**

3. **Coagulation Test.**—Place a small amount of the protean in a test-tube, add a little water and boil. Now add dilute hydrochloric acid and note that the protein no longer dissolves. It has been coagulated.

4. **Tests on Edestan Solution.**—Dissolve the remainder of the edestan precipitate in 0.2 per cent hydrochloric acid and make the following tests:

(*a*) *Biuret Test.*

(*b*) *Influence of Protein Precipitants.*—Try a few protein precipitants such as *picric acid* and *mercuric chloride.*

METAPROTEINS.

The metaproteins are formed from the native simple proteins through an action similar to that by which proteans are formed. In the case of the *metaproteins*, however, the changes in the original protein molecule are more profound. These derived proteins are characterized by being soluble in very weak acids and alkalis, but *insoluble in neutral fluids.* The metaproteins have generally been termed *albuminates*, but inasmuch as the termination *ate* signifies *a salt* it has always been somewhat of a misnomer.

Two of the principal metaproteins are the *acid metaprotein* or so-called acid albuminate and the *alkali metaprotein* or so-called alkali albuminate. They differ from the native simple proteins principally in being insoluble in sodium chloride solution and in not being coagulated *except when suspended in neutral fluids.* Both forms of metaprotein are precipitated upon the approximate neutralization of their solutions. They are precipitated by saturating their solutions with ammonium sulphate, and by sodium chloride, also, provided they are dissolved in an acid solution. Acid metaprotein contains a higher percentage of nitrogen and sulphur than the alkali metaprotein from the same source, since some of the nitrogen and sulphur of the original protein is liberated in the formation of the latter. Because of this fact, it is

[1] The edestan solution preserved from experiment (5), page 102, may be used.

impossible to transform an alkali metaprotein into an acid metaprotein, while it is possible to reverse the process and transform the acid metaprotein into the alkali modification.

Experiments on Metaproteins.

ACID METAPROTEIN (ACID ALBUMINATE).

Preparation and Study.—Take 25 grams of hashed lean beef washed free from the major portion of blood and inorganic matter, and place it in a medium-sized beaker with 100 c.c. of 0.2 per cent HCl. Place it on a boiling water-bath for one-half hour, filter, *cool*, and divide the filtrate into two parts. Neutralize the *first part* with *dilute* KOH solution, filter off the precipitate of *acid metaprotein* and make the following tests:

(1) *Solubility.*—Solubility in the ordinary solvents (see page 22).

(2) *Millon's Reaction.*

(3) *Coagulation Test.*—Suspend a little of the metaprotein in water (neutral solution) and heat to boiling for a few moments. Now add 1–2 drops of KOH solution to the water and see if the metaprotein is still soluble in dilute alkali. What is the result and why?

(4) *Test for Loosely Combined Sulphur* (see page 100).

Subject the *second part* of the original solution to the following tests:

(1) *Coagulation Test.*—Heat some of the solution to boiling in a test-tube. Does it coagulate?

(2) *Biuret Test.*

(3) *Influence of Protein Precipitants.*—Try a few protein precipitants such as *picric acid* and *mercuric chloride.* How do the results obtained compare with those from the experiments on egg albumin? (See page 95.)

ALKALI METAPROTEIN (ALKALI ALBUMINATE).

Preparation and Study.—Carefully separate the white from the yolk of a hen's egg and place the former in an evaporating dish. Add concentrated potassium hydroxide solution, *drop by drop,* stirring continuously. The mass gradually thickens and finally assumes the consistency of jelly. This is *solid alkali metaprotein* or "Lieberkühn's jelly." Do not add an excess of potassium hydroxide or the jelly will dissolve. Cut it into small pieces, place a cloth or wire gauze over the dish, and by means of running water wash the pieces free from adherent alkali. Now add a small amount of water, which forms a weak alkaline solution with the alkali within the pieces, and dissolve

the jelly by gentle heat. *Cool* the solution and divide it into two parts. Proceed as follows with the *first part:* Neutralize with *dilute* hydrochloric acid, noting the odor of the liberated hydrogen sulphide as the alkali metaprotein precipitates. Filter off the precipitate and test as for acid metaprotein, page 108, noting particularly the sulphur test. How does this test compare with that given by the acid metaprotein? Make tests on the *second part* of the solution the same as for acid metaprotein, page 108.

Coagulated Proteins.

These derived proteins are produced from unaltered protein materials by heat, by long standing under alcohol, or by the continuous movement of their solutions such as that produced by rapid stirring or shaking. In particular instances, such as the formation of fibrin from fibrinogen (see page 187), the coagulation may be produced by enzyme action. Ordinary soluble proteins after having been transformed into the coagulated modification are no longer soluble in the ordinary solvents. Upon being heated in the presence of strong acids or alkalis, coagulated proteins are converted into metaproteins.

Many proteins coagulate at an approximately fixed temperature under definite conditions (see pp. 98 and 231). This characteristic may be applied to separate different coagulable proteins from the same solution by fractional coagulation. The coagulation temperature frequently may serve in a measure to identify proteins in a manner similar to the melting-point or boiling-point of many other organic substances. The separation of proteins by fractional coagulation is thus analogous to the separation of volatile substances by means of *fractional distillation.* This method of separating proteins is not a satisfactory one, however, inasmuch as proteins in solution have different effects upon one another and also because of the fact that the nature of the solvent causes a variation in the temperature at which a given protein coagulates. The nature of the process involved in the coagulation of proteins by heat is not well understood, but it is probable that in addition to the altered arrangement of the component atoms in the molecule, there is a mild hydrolysis which is accompanied by the liberation of minute amounts of hydrogen, nitrogen, and sulphur. The presence of a neutral salt or a trace of a mineral acid may facilitate the coagulation of a protein solution (see page 98), whereas any appreciable amount of acid or alkali will retard or entirely prevent such coagulation.

Experiments on Coagulated Protein.

Ordinary coagulated egg-white may be used in the following tests:

1. **Solubility.**—Try the solubility of *small* pieces of the coagulated protein in each of the ordinary solvents (see page 22).

2. **Millon's Reaction.**

3. **Xanthoproteic Reaction.**—*Partly* dissolve a medium-sized piece of the protein in concentrated nitric acid. *Cool* the solution and add an excess of ammonium hydroxide. Both the protein solution and the undissolved protein will be colored orange.

4. **Biuret Test.**—*Partly* dissolve a medium-sized piece of the protein in concentrated potassium hydroxide solution. If the proper dilution of cupric sulphate solution is now added the white coagulated protein, as well as the protein solution, will assume the characteristic purplish-violet color.

5. **Hopkins-Cole Reaction.**—Conduct this test according to the modification given on page 99.

Secondary Protein Derivatives.

These derivatives result from a more profound cleavage of the protein molecule than that which occurs in the formation of the primary derivatives. The class includes *proteoses*, *peptones*, and *peptides*.

PROTEOSES AND PEPTONES.

Proteoses are intermediate products in the digestion of proteins by proteolytic enzymes, as well as in the decomposition of proteins by hydrolysis and the putrefaction of proteins through the action of bacteria. Proteoses are called also *albumoses* by some writers, but it seems more logical to reserve the term albumose for the proteose of albumin.

Peptones are formed after the proteoses and it has been customary to consider them as the last product of the processes before mentioned which still possess true protein characteristics. In other words it has been considered that the protein nature of the end-products of the cleavage of the protein molecule ceased with the peptones, and that the simpler bodies formed from peptones were substances of a different nature (see page 65). However, as the end-products have been more carefully studied, it has been found to be no easy matter to designate the exact character of a peptone or to indicate the exact point at which the

peptone characteristic ends and the *peptide* characteristic begins. The situation regarding the proteoses, peptones and peptides, is at present a most unsatisfactory one because of the unsettled state of our knowledge regarding them. The exact differences between certain members of the peptone and peptide groups remain to be more accurately established. It has been quite well established that the peptones are peptides or mixtures of peptides, but the term peptide is used at present to designate only those possessing a definite structure.

There are several proteoses (protoproteose, heteroproteose and deuteroproteose), and at least two peptones (amphopeptone and antipeptone), which result from proteolysis. The differentiation of the various proteoses and peptones at present in use is rather unsatisfactory. These compounds are classified according to their varying solubilities, especially in ammonium sulphate solutions of different strengths. The exact differences in composition between the various members of the group remain to be more accurately established. Because of the difficulty attending the separation of these bodies, pure proteose and peptone are not easy to procure. The so-called peptones sold commercially contain a large amount of proteose. As a class the proteoses and peptones are very soluble, diffusible bodies which are non-coagulable by heat. *Peptones differ from proteoses in being more diffusible, non-precipitable by* $(NH_4)_2SO_4$, *and by their failure to give any reaction with potassium ferrocyanide and acetic acid, potassio-mercuric iodide and* HCl, *picric acid, and trichloracetic acid.* The so-called *primary proteoses* are precipitated by HNO_3 and are the only members of the proteose-peptone group which are so precipitated.

Some of the more general characteristics of the proteose-peptone group may be noted by making the following simple tests on a proteose-peptone powder:

(1) *Solubility.*—Solubility in the ordinary solvents (see page 22).

(2) *Millon's Reaction.*

Dissolve a little of the powder in water and test the solution as follows:

(1) *Precipitation by Picric Acid.*—To 5 c.c. of proteose-peptone solution in a test-tube add picric acid until a permanent precipitate forms. The precipitate disappears on heating and returns on cooling.

(2) *Precipitation by a Mineral Acid.*—Try the precipitation by nitric acid.

(3) *Coagulation Test.*—Heat a little proteose-peptone solution to boiling. Does it coagulate like the other simple proteins studied?

SEPARATION OF PROTEOSES AND PEPTONES.[1]

Place 50 c.c. of proteose-peptone solution in an evaporating dish or casserole, and *half-saturate* it with ammonium sulphate solution, which may be accomplished by adding an equal volume of *saturated* ammonium sulphate solution. At this point note the appearance of a precipitate of the *primary proteoses* (protoproteose and heteroproteose). Now heat the half-saturated solution and its suspended precipitate to boiling and *saturate* the solution with *solid* ammonium sulphate. At full saturation the *secondary proteoses* (deuteroproteoses) are precipitated. The peptones remain in solution.

Proceed as follows with the precipitate of proteoses: Collect the sticky precipitate on a rubber-tipped stirring rod or remove it by means of a watch glass to a small evaporating dish and dissolve it in a little water. To remove the ammonium sulphate, which adhered to the precipitate and is now in solution, add barium carbonate, boil, and filter off the precipitate of barium sulphate. Concentrate the proteose solution to a small volume[2] and make the following tests:

(1) *Biuret Test.*

(2) *Precipitation by Nitric Acid.*—What would a precipitate at this point indicate?

(3) *Precipitation by Trichloracetic Acid.*—This precipitate dissolves on heating and returns on cooling.

(4) *Precipitation by Picric Acid.*—This precipitate also disappears on heating and returns on cooling.

(5) *Precipitation by Potassio-mercuric Iodide and Hydrochloric Acid.*

(6) *Coagulation Test.*—Boil a little in a test-tube. Does it coagulate?

(7) *Acetic Acid and Potassium Ferrocyanide Test.*

The solution containing the peptones should be cooled and filtered, and the ammonium sulphate in solution removed by boiling with barium carbonate as described above. After filtering off the barium sulphate precipitate, concentrate the peptone filtrate to a small volume and repeat the test as given under the proteose solution, above. In the biuret test the solution should be made very strongly alkaline with *solid* potassium hydroxide.

[1] The separation of proteoses and peptones by means of fractional precipitation with ammonium sulphate does not possess the significance it once possessed inasmuch as the boundary between these substances and *peptides* is not well defined (see p. 110).

[2] If the proteoses are desired in powder form, this concentrated proteose solution may now be precipitated by alcohol, and this precipitate, after being washed with absolute alcohol and with ether, may be dried and powdered.

In the case of Albumin the more you heat a ppt., the denser they get. The proteoses' ppts. dissolve on heating.

PEPTIDES.

The peptides are "definitely characterized combinations of two or more amino acids, the carboxyl (COOH) group of one being united with the amino (NH_2) group of the other with the elimination of a molecule of water." These peptides are more fully discussed on pages 65 and 111.

REVIEW OF PROTEINS.

In order to facilitate the student's review of the proteins, the preparation of a chart similar to the model given is recommended. The signs + and − may be conveniently used to indicate positive and negative reactions.

MODEL CHART FOR REVIEW PURPOSES.

Protein.	Solubility.						Precipitation Tests.								Salting-out Tests.		Diffusion.	Coagulation by Heat.
	Water.	10% NaCl.	0.2% HCl.	0.5% Na_2CO_3.	Conc. HCl.	Conc. KOH.	Protein Color Test.	Mineral Acid (HNO_3).	Metallic Salt ($HgCl_2$).	Alcohol.	Pot. Ferrocyanide + Acetic Acid.	Potassio-mercuric Iodide + HCl.	Picric Acid.	Trichloracetic Acid.	$(NH_4)_2SO_4$.	NaCl.		
Albumin.	S						+	+	+	+	+	+	+	+	−			+
Globulin.	−	S	−	−	S	−	+	+	+	+	+	+	+	+	+	+		+
Protean.																		
Acid metaprotein.	S	−	S	S	S	ppt	+	+	+	−	+	+	+	+	+	+		+
Alkali metaprotein.	S		−		ppt	S	+	+	+	−	+	+	+	+	+			+
Proteose.	S						+	−			+	+	+	+	+			−
Peptone.	S						−	−			−	−	−	−				
Coagulated protein.	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−		

"UNKNOWN" MIXTURES AND SOLUTIONS OF PROTEINS.

At this point the student's knowledge of the characteristics of the various proteins studied will be tested by requiring him to examine several "unknown" protein mixtures or solutions and make full report upon the same. The scheme given on page 114 may be used in this examination.

SCHEME FOR THE DETECTION OF PROTEINS.

If the solution is acid or alkaline it should be *approximately* neutralized.
If a precipitate forms it should be filtered off. The neutralization need not necessarily proceed until an exact neutral reaction is obtained but should cease at the point where the largest precipitate is secured.

Precipitate indicates.

Acid metaprotein or *proteān*, if the unknown solution was acid in reaction. (Test the precipitate by protein color tests.) It is practically impossible, in the light of our present knowledge, to differentiate between the *proteans* and the *metaproteins*.

Alkali metaprotein, mucin (*mucoid*) or *phosphoprotein*, if the unknown solution was alkaline in reaction.

Alkali metaprotein, if the precipitate is *very easily* soluble in an excess of acid. (Test the metaprotein by protein color tests.)

Phosphoprotein, if the precipitate is *fairly* soluble in an excess of acid. (Test the precipitate by protein color tests. Also test the precipitate for phosphorus by test on page 247.)

Mucin or *mucoid*, if the precipitate is soluble with difficulty in an excess of acid. Hydrolyze some of the precipitate and test by Fehling's solution. See page 27.

Filtrate. This may contain *albumin, globulin, proteose, peptone*, and *gelatin*.

Neutralize the filtrate, heat to boiling, and acidify slightly. If there is any coagulation note the temperature at which it occurs. Filter off the coagulum.

Coagulum indicates *albumin* or *globulin* or both. Test by protein color tests.)

Remove the metaprotein from some of the unknown solution, if any is present, and *half-saturate* the filtrate with $(NH_4)_2SO_4$. (By adding an equal volume of *saturated* $(NH_4)_2SO_4$ solution.) If a precipitate forms, filter it off.

Precipitate indicates *globulin* or primary proteose. (Try protein color tests.)

Differentiate between globulin and primary proteose by pouring some of the unknown solution into 20–40 volumes of water.

Precipitate indicates *globulin*.

No precipitate indicates *primary proteose*.

Filtrate. *Saturate* with *solid* $(NH_4)_2SO_4$. Precipitate indicates gelatin, secondary proteose, or albumin. Filter it off and dissolve in *warm* water.

Heat to boiling.

Coagulation indicates *albumin*.

No coagulation indicates *gelatin* or secondary proteose.

Saturate this neutral solution with $MgSO_4$.

Precipitate indicates *gelatin*.

Secondary proteose may be present in the filtrate.

Filtrate. *Half-saturate* with $(NH_4)_2SO_4$.

Precipitate indicates *primary proteose*.

Filtrate. *Saturate hot* with $(NH_4)_2SO_4$

Precipitate indicates *secondary* proteose, or *gelatin*.

Dissolve the precipitate in warm water[1] and saturate this neutral solution with $MgSO_4$.

Precipitate indicates *gelatin*. (Try any tests given on page 226.)

Filtrate. Cool and filter off the $(NH_4)_2SO_4$.

Test for peptone by the biuret test. (Use an excess of *solid* KOH.)

Filtrate may contain *secondary proteose*. (Try any tests given on page 112.)

[1] We may also differentiate between gelatin and proteose by means of the Hopkins-Cole reaction (see page 89). A positive reaction here would indicate proteose and a negative reaction would indicate gelatin.

CHAPTER VI.

GASTRIC DIGESTION.

GASTRIC digestion takes place in the stomach and is promoted by the gastric juice, which is secreted by the glands of the stomach mucosa. These glands are of two kinds, fundus glands and pyloric glands which are situated, as their names imply, in the regions of the fundus and pylorus. The principal foods acted upon in gastric digestion are the proteins which are so changed by its processes as to become better prepared for further digestion in the intestine and for their final absorption.

From reliable experiments made upon lower animals it is evident that the gastric juice is secreted as the result of stimuli of two forms, *i. e.*, *psychical* stimuli and *chemical* stimuli. The psychical form of stimuli may be produced by the sight, thought, or taste of food, and the chemical stimuli may be produced by certain substances, such as water, the extractives of meat, etc., when coming in contact with the stomach mucosa. The volume of gastric juice secreted during any given period of digestion, varies with the quantity and kind of the food. These conclusions were deduced principally from a series of so-called *delusive feeding* experiments. A dog was prepared with two œsophageal openings and a gastric fistula. When thus prepared and fed foods of various kinds such as meat and bread, the material instead of passing to the stomach, would invariably find its way out of the animal's body at the upper œsophageal opening. Through the medium of the gastric fistula the course of the secretion of gastric juice could be carefully followed. It was found that when the dog ate meat, for example, there was a large secretion of gastric juice notwithstanding no portion of the food eaten had reached the stomach. Further experiments made through the medium of a *cul-de-sac* formed from the stomach wall have given us many valuable conclusions, among others those regarding the influence of the chemical stimuli. The method followed was to feed the animal certain substances and note the secretion of gastric juice in the miniature stomach while the real process of digestion was taking place in the stomach proper.

Normal gastric juice is a thin, light colored fluid which is acid

in reaction and has a specific gravity varying between 1.001 and 1.010. It contains only 2–3 per cent of solid matter which is made up principally of hydrochloric acid, sodium chloride, potassium chloride, earthy phosphates, mucin and the enzymes *pepsin*, *gastric rennin*, and *gastric lipase;* the hydrochloric acid and the enzymes are of the greatest importance. The acidity of the gastric juice is due to *free* hydrochloric acid which is secreted by the parietal cells of the fundus as well as by the chief cells of both the fundus and pyloric glands, and, in man, is generally present to the extent of 0.2–0.3 per cent. When the amount of hydrochloric acid varies to any considerable degree from these values a condition of hypoacidity or hyperacidity is established. Hydrochloric acid has the power of combining with protein substances taken in the food, thus forming so-called *combined* hydrochloric acid. This combined acid is a less potent germicide than *free* hydrochloric acid and has less power to destroy the amylolytic enzyme *salivary amylase* (*ptyalin*) of the saliva. This last fact explains to a degree the possibility of the continuance of salivary digestion in the stomach. The hydrochloric acid of the gastric juice forms a medium in which the pepsin can most satisfactorily digest the protein food, and at the same time it acts as an antiseptic or germicide which prevents putrefactive processes in the stomach. It also possesses the power of inverting cane sugar, this property being due to the hydrogen ion. When the hydrochloric acid of the gastric juice is diminished in quantity (hypoacidity) or absent, as it may be in many cases of functional or organic disease, there is no check to the growth of micro-organisms in the stomach. There are, however, certain of the more resistant spores which even the normal acidity of the gastric juice will not destroy. A condition of hypoacidity may also give rise to fermentation with the formation of comparatively large amounts of such substances as lactic acid and butyric acid.

The question of the origin of the hydrochloric acid of the gastric juice is a problem to whose solution many investigators have given much attention. Many theories have been proposed, among them being Bunge's *mass action theory*, Köppe's *electrolytic dissociation theory*, and the more recent theory based upon the *interaction of sodium chloride and lactic acid*. We cannot go into a discussion of these various theories. Each of them has met with objection and we have, as yet, no generally accepted theory as to the origin of the hydrochloric acid of the gastric juice. That this hydrochloric acid originates from the chlorides of the blood is apparently a well established fact, but farther than this no positive statement can be made.

The most important of the enzymes of the gastric juice is the proteolytic enzyme *pepsin*. The pepsin does not originate as such in the gastric cells but is formed from its precursor, the *zymogen* or mother-substance pepsinogen, which is produced by the parietal cells of the fundus as well as by the chief cells of the fundus and pyloric glands. Upon coming in contact with the hydrochloric acid of the secretion this pepsinogen is immediately transformed into pepsin. Pepsin is not active in alkaline or neutral solutions but requires at least a faint acidity before it can exert its power to dissolve and digest proteins. The percentage of hydrochloric acid facilitating the most rapid peptic action varies with the character of the protein acted upon, *e. g.*, 0.08 per cent to 0.1 per cent for the digestion of fibrin and 0.25 per cent for the digestion of coagulated egg-white. While hydrochloric acid is the acid usually employed to promote artificial peptic proteolysis, other acids, organic and inorganic, will serve the same purpose. Acidity of the liquid is necessary to promote the activity of the pepsin, but the acidity need not necessarily be confined to hydrochloric acid.

In common with many other enzymes pepsin acts best at about 38°–40° C. and its digestive power decreases as the temperature is lowered, the enzyme being only slightly active at 0° C. Its power is only temporarily inhibited by the application of such low temperatures, however, and the enzyme regains its full proteolytic power upon raising the temperature to 40° C. As the temperature of a digestive mixture is raised above 40° C. the pepsin gradually loses its activity until at about 80°–100° C. its proteolytic power is permanently destroyed.

Our ideas regarding the nature of the products formed in the course of peptic proteolysis have undergone considerable revision in recent years. The former view that these products included only acid albuminate (acid metaprotein), proteoses and peptones is no longer tenable. From the investigations of numerous observers we have learned that artificial gastric digestion *if permitted to proceed for a sufficiently long period* will yield, in addition to proteoses and peptones, a long list of protein cleavage products which are crystalline in character, including *leucine*, *tyrosine*, *alanine*, *phenylalanine*, *aspartic acid*, *glutamic acid*, *proline*, *leucinimide*, *valine*, and *lysine*. A similar group of substances may result from the action of the enzyme trypsin (see p. 138). The relative amounts of proteoses, peptones, and crystalline substances formed depends to a great extent upon the character of the protein undergoing digestion, *e. g.*, a greater proportion of proteoses results from the digestion of fibrin than from the digestion of

coagulated egg-white. We must not be led into the error of thinking that the large number of protein cleavage products just mentioned are formed in the course of normal gastric digestion *within the animal organism.* They are formed only after comparatively long-continued hydrolysis. In pancreatic digestion, however, there are formed even under normal conditions, the large number of cleavage products to which reference has been made. Peptic proteolysis, therefore, within the animal organism differs from tryptic proteolysis (see page 138) in that the former yields larger amounts of proteoses, smaller amounts of peptones and no considerable quantity of crystalline bodies as end-products in the brief period during which proteins are ordinarily subjected to gastric digestion. Prolonged hydrolysis with gastric juice does, however, yield considerable quantities of the non-protein end-products.

Gastric rennin, the second enzyme of the gastric juice, is what is known as a *milk curdling* or *protein coagulating* enzyme. Rennin acts upon the caseinogen of the milk, splitting it into a proteose-like body and soluble casein. This soluble body, in the presence of calcium salts, combines with calcium, forming *calcium casein* or true *casein* which is insoluble and precipitates. There is some uncertainty regarding the reaction to litmus in which gastric rennin shows the greatest activity. It is, however, said to be active in neutral, alkaline, or acid solution. However, it probably possesses its greatest activity in the presence of a slight acid reaction, as would naturally be expected. It is especially abundant in the gastric mucosa of the calf, and is used to curdle the milk used in cheese-making. Gastric rennin is always present normally in the gastric juice but in certain pathological conditions such as atrophy of the mucosa, chronic catarrh of the stomach, or in carcinoma it may be absent.

The theory that the proteolytic activity and the milk curdling property of the gastric juice reside in a single molecule is causing much controversy at the present time. The theory was originally advanced by the Pawlow school.

Gastric lipase, the third enzyme of the gastric juice, is a fat-splitting enzyme. It possesses but slight activity when the gastric juice is of normal acidity, but evinces its action principally at such times as a gastric juice of low acidity is secreted either from physiological or pathological cause. The digestion of fat in the stomach is, however, at most, of but slight importance as compared with the digestion of fat in the intestine through the action of the lipase of the pancreatic juice (see page 140).

PREPARATION OF AN ARTIFICIAL GASTRIC JUICE.

Dissect the mucous membrane of a pig's stomach from the muscular portion and discard the latter. Divide the mucous membrane into two parts (4/5 and 1/5). Cut up the larger portion, place it in a large-sized beaker with 0.4 per cent hydrochloric acid and keep at 38°–40° C. for at least 24 hours. Filter off the residue, consisting principally of nuclein and anti-albumid, and use the filtrate as an artificial gastric juice. This filtrate contains pepsin, rennin, and the products of the digestion of the stomach tissue, *i. e.*, acid metaprotein (acid albuminate), proteoses, peptones, etc.

Preparation of a Glycerol Extract of Pig's Stomach.

Take the one-fifth portion of the mucous membrane of the pig's stomach not used in the preparation of the artificial gastric juice, cut it up very finely, place it in a small-sized beaker and cover the membrane with glycerol. Stir frequently and allow to stand at room temperature for at least 24 hours. The glycerol will extract the *pepsinogen*. Separate, with a pipette or by other means, the glycerol from the pieces of mucous membrane and use the glycerol extract as required in the later experiments.

Products of Gastric Digestion.

Into the artificial gastric juice, prepared as above described, place the protein material (fibrin, coagulated egg-white, or lean beef) provided for you by the instructor, add 0.4 per cent hydrochloric acid as suggested by the instructor and keep the digestion mixture at 40° C. for 2 to 3 days. Stir frequently and keep *free* hydrochloric acid present in the solution. (For tests for free hydrochloric acid see p. 120).

The original protein has been digested and the solution now contains the products of peptic proteolysis, *i. e.*, acid metaprotein (acid albuminate), proteoses, peptones, etc. The insoluble residue may include nuclein and anti-albumid. Filter the digestive mixture and after testing for *free* hydrochloric acid neutralize the filtrate with potassium hydroxide solution. If any of the acid metaprotein (acid albuminate) is still untransformed into proteoses it will precipitate upon neutralization. If any precipitate forms heat the mixture to boiling, and filter. If no precipitate forms proceed without filtering.

We now have a solution containing a mixture consisting principally of proteoses and peptones. Separate and identify the proteoses and peptones according to the directions given on pages 110 and 112.

Tests for Free and Combined HCl.

These tests are made with a class of reagents known as *indicators*, so called because they serve to indicate the nature of the reaction of a solution. These indicators are weak acids or bases and are but slightly dissociable. The dissociation, with the formation of the colored ion, forms the basis for the color reaction.

Examine each of the following solutions by means of the tests given below and report the results in a form similar to the chart given on page 122: (1) 0.2 per cent *free* hydrochloric acid. (2) 0.05 per cent *free* hydrochloric acid. (3) 0.01 per cent *free* hydrochloric acid. (4) 0.05 per cent *combined* hydrochloric acid. (5) 1 per cent lactic acid. (6) Equal volumes of 0.2 per cent *free* hydrochloric acid and 1 per cent lactic acid. (7) 1 per cent potassium hydroxide.

1. **Dimethyl-amino-azobenzene (or Töpfer's Reagent),**[1]

$$N(CH_3)_2-C_6H_4-N=N-C_6H_5.$$

Place 1–2 drops of the reagent in the solution to be tested. Free mineral acid (hydrochloric acid) is indicated by the production of a pinkish-red color. If free acid is absent a yellow color ordinarily results.

2. **Günzberg's Reagent.**[2]—Place 1–2 drops of the reagent in a small porcelain evaporating dish and *carefully* evaporate to dryness over a *low* flame. Insert a glass stirring rod into the mixture to be tested and draw the moist end of the rod through the dried reagent. Warm again gently and note the production of a purplish-red color in the presence of *free* hydrochloric acid.

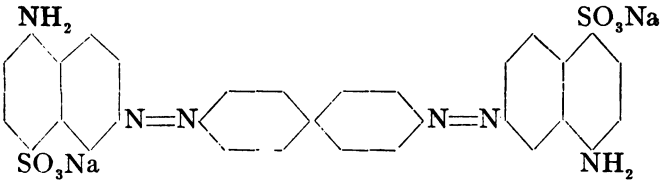
3. **Boas' Reagent.**[3]—Perform this test in the same manner as 2, above. Free hydrochloric acid is indicated by the production of a rose-red color which becomes less pronounced on cooling.

[1] To prepare Töpfer's reagent dissolve 0.5 gram of di-methyl-amino-azobenzene in 100 c.c. of 95 per cent alcohol.

[2] Günzberg's reagent is prepared by dissolving 2 grams of phloroglucin and 1 gram of vanillin in 100 c.c. of 95 per cent alcohol.

[3] Boas' reagent is prepared by dissolving 5 grams of resorcin and 3 grams of sucrose in 100 c.c. of 95 per cent alcohol.

4. **Congo Red,**[1]



Conduct this test according to the directions given under 1 or 2, page 120. A blue color indicates free hydrochloric acid, a violet color indicates an organic acid and a brown color indicates combined hydrochloric acid. Congo-red paper, made by immersing ordinary filter paper in the indicator and subsequently drying, may be used in this test.

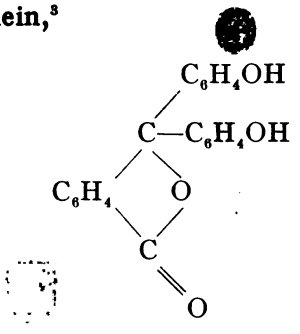
5. **Tropæolin OO,**[2]

$$NH(C_6H_5)-C_6H_4-N=N-C_6H_4-SO_3Na.$$

Place 2 drops of the solution to be tested and 1 drop of the indicator in an evaporating dish and evaporate to dryness over a low flame. The formation of a reddish-violet color indicates *free* hydrochloric acid.

This test may also be conducted in the same manner as 2, page 120.

6. **Phenolphthalein,**[3]



Add the indicator directly to the solution, or apply the test according to the directions given under 2 on page 120. This indicator serves to denote the *total acidity* since it is acted upon by free mineral acids, combined acids, organic acids, and acid salts. A red color indicates the

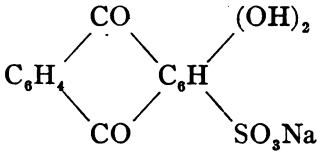
[1] This indicator is prepared by dissolving 0.5 gram of congo red in 90 c.c. of water and adding 10 c.c. of 95 per cent alcohol.

[2] Prepared by dissolving 0.05 gram of tropæolin OO in 100 c.c. of 50 per cent alcohol.

[3] This indicator is prepared by dissolving 1 gram of phenolphthalein in 100 c.c. of 95 per cent alcohol.

presence of an alkali and the indicator is colorless in the presence of a neutral or acid reaction. This indicator is unsatisfactory in the presence of ammonia.

7. **Sodium Alizarin Sulphonate,**[1]



This indicator may be used directly in the solution to be tested, or the test may be applied as 2, page 120. It serves to indicate all acid reactions except those due to *combined* acids. A reddish-violet color indicates an alkaline reaction, while a yellow color indicates an acid reaction due to a free mineral acid, an organic acid, or an acid salt.

Report the results of your tests tabulated in the form given below:

	Solutions Examined.						
Name of Indicator.	0.2% HCl.	0.05% HCl.	0.01% HCl.	0.05% Combined HCl.	1% Lactic Acid.	Equal Vols. 0.2% HCl and 1% Lactic Acid.	1% KOH.
Töpfer's Reagent.							
Günzberg's Reagent							
Boas' Reagent.							
Congo Red.							
Tropæolin OO.							
Phenolphthalein.							
Alizarin.							

GENERAL EXPERIMENTS ON GASTRIC DIGESTION.

1. **Conditions Essential for the Action of Pepsin.**—Prepare four test-tubes as follows:

(*a*) Five c.c. of pepsin solution.

(*b*) Five c.c. of 0.4 per cent hydrochloric acid.

(*c*) Five c.c. of pepsin-hydrochloric acid solution.

(*d*) Two or three c.c. of pepsin solution and 2–3 c.c. of 0.5 per cent sodium carbonate solution.

[1] Prepare this indicator by dissolving 1 gram of sodium alizarin sulphonate in 100 c.c. of water.

Introduce into each tube a small piece of fibrin and place them on the water-bath at 40° C. for one-half hour, carefully noting any changes which occur.[1] Now combine the contents of tubes (*a*) and (*b*) and see if any further change occurs after standing at 40° C. for 15–20 minutes. Explain the results obtained from these five experiments.

2. **Influence of Different Temperatures.**—In each of four test-tubes place 5 c.c. of pepsin-hydrochloric acid solution. Immerse one tube in cold water from the faucet, keep a second tube at room temperature and place a third on the water-bath at 40° C. Boil the contents of the fourth tube for a few moments, then cool and also keep it at 40° C. Into each tube introduce a small piece of fibrin and note the progress of digestion. In which tube does the most rapid digestion occur? Explain this.

3. **The Most Favorable Acidity.**—Prepare three tubes as follows:

(*a*) Five c.c. of 0.2 per cent pepsin-hydrochloric acid solution.

(*b*) Two or three c.c. of 0.2 per cent hydrochloric acid + 1 c.c. of concentrated hydrochloric acid + 5 c.c. of pepsin solution.

(*c*) One c.c. of 0.2 per cent pepsin-hydrochloric acid solution + 5 c.c. of water.

Introduce a small piece of fibrin into each tube, keep them at 40° C., and note the progress of digestion. In which degree of acidity does the fibrin digest the most rapidly?

4. **Differentiation Between Pepsin and Pepsinogen.**—Prepare five tubes as follows:

(*a*) Few drops of glycerol extract of pepsinogen + 2–3 c.c. of water.

(*b*) Few drops of glycerol extract of pepsinogen + 5 c.c. of 0.2 per cent hydrochloric acid.

(*c*) Few drops of glycerol extract of pepsinogen + 5 c.c. of 0.5 per cent sodium carbonate.

(*d*) Two or three c.c. of pepsin solution + 2–3 c.c. of 1 per cent sodium carbonate.

(*e*) Few drops of glycerol extract of pepsinogen + 5 c.c. of 1 per cent sodium carbonate.

Add a small piece of fibrin to the contents of each tube, keep the five tubes at 40° C. for one-half hour and observe any changes

[1] Digestion of fibrin in a pepsin-hydrochloric acid solution is indicated first by a *swelling* of the protein due to the action of the acid, and later by a *disintegration* and *dissolving* of the fibrin due to the action of the pepsin-hydrochloric acid. If uncertain at any time whether digestion has taken place, the solution under examination may be filtered and the biuret test applied to the filtrate. A positive reaction will signify the presence of acid metaprotein (acid albuminate), proteoses (albumoses), or peptones, the presence of any one of which would indicate that digestion has taken place.

which may have occurred. To (*a*) add an equal volume of 0.4 per cent hydrochloric acid, neutralize (*c*), (*d*) and (*e*) with hydrochloric acid and add an equal volume of 0.4 per cent hydrochloric acid. Place these tubes at 40° C. again and note any further changes which may occur. What contrast do we find in the results from the last three tubes? Why is this so?

5. **Comparative Digestive Power of Pepsin with Different Acids.**—Prepare a series of tubes each containing one of the following acids: 0.5 per cent acetic, lactic, oxalic, salicylic, tannic, and butyric, and 0.2 per cent hydrochloric, sulphuric, nitric, arsenious, and *combined* hydrochloric. To each acid add a few drops of the glycerol extract of pig's stomach and a small piece of fibrin. Shake well, place at 40° C., and note the progress of digestion. In which tubes does the most rapid digestion occur?

6. **Influence of Metallic Salts, etc.**—Prepare a series of tubes and into each tube introduce 4 c.c. of pepsin-hydrochloric acid solution and 1/2 c.c. of one of the chemicals listed in Experiment 18 under Salivary Digestion, page 58. Introduce a small piece of fibrin into each of the tubes and keep them at 40° C. for one-half hour. Note the variations in the progress of digestion. Where has the least rapid digestion occurred?

7. **Sahli's Desmoid Reaction.**—This is a method for testing gastric function without using the stomach tube. The underlying principle of the test is the fact that raw catgut may be digested in gastric juice but is entirely indigestible in pancreatic juice. The test is made as follows: A methyleneblue pill is introduced into a small rubber bag and the mouth of the bag subsequently tied with catgut.[1] The small bag is then ingested immediately after the mid-day meal and the urine examined 5, 7, 9 and 18–20 hours later for methylene blue. If methylene blue is present in appreciable quantity, it will impart to the urine a greenish-blue color. If not present in sufficient amount to impart this color the urine should be boiled with 1/5 its volume of glacial acetic acid, whereupon a greenish-blue color results if the chromogen of methylene blue is present. This contingency seldom arises, however, inasmuch as in most cases of uncolored urine it will be found that the rubber bag has passed through the stomach

[1] About 0.05 gram of methylene blue is mixed with sufficient *ext. glycyrrhizæ* to form a pill about 3–4 mm. in diameter. The pill is then placed in the center of a square piece of thin rubber dam and a little bag-like receptacle constructed by a twisting movement. The neck of the bag is then closed by wrapping three turns of catgut about it. The most satisfactory catgut to use is *number* 00 *raw catgut* which has previously been soaked in water until soft. When ready for use the bag should sink instantly when placed in water and be water-tight.

unopened. It the methylene blue is found in the urine inside of 18–20 hours a satisfactory gastric function is indicated.

8. **Testing the Motor and Functional Activities of the Stomach.**—This test is performed the same as Experiment 19 under Salivary Digestion, page 58. If the experiment was carried out under salivary digestion it will not be necessary to repeat it here.

9. **Influence of Bile.**—Prepare five tubes as follows:

(*a*) Five c.c. of pepsin-hydrochloric acid solution + 1/2–1 c.c. of bile.

(*b*) Five c.c. of pepsin-hydrochloric acid solution + 1–2 c.c. of bile.

(*c*) Five c.c. of pepsin-hydrochloric acid solution + 2–3 c.c. of bile.

(*d*) Five c.c. of pepsin-hydrochloric acid solution + 5 c.c. of bile.

(*e*) Five c.c. of pepsin-hydrochloric acid solution.

Introduce into each tube a small piece of fibrin. Keep the tubes at 40° C. and note the progress of digestion. Does the bile exert any appreciable influence? How?

10. **Influence of Gastric Rennin on Milk.**—Prepare a series of five tubes as follows:

(*a*) Five c.c. of fresh milk + 0.2 per cent hydrochloric acid (add slowly until precipitate forms).

(*b*) Five c.c. of fresh milk + 5 drops of rennin solution.

(*c*) Five c.c. of fresh milk + 10 drops of 0.5 per cent sodium carbonate solution.

(*d*) Five c.c. of fresh milk + 10 drops of a saturated solution of ammonium oxalate.

(*e*) Five c.c. of fresh milk + 5 drops of 0.2 per cent hydrochloric acid. Now to each of the tubes (*c*), (*d*), and (*e*) add 5 drops of rennin solution. Place the whole series of five tubes at 40° C. and after 10–15 minutes note what is occurring in the different tubes. Give a reason for each particular result.

11. **Tests for Lactic Acid.**—(*a*) *Uffelmann's Reaction.*—To a small quantity of Uffelmann's reagent[1] in a test-tube add a few drops of a lactic acid solution. The amethyst-blue color of the reagent is displaced by a straw yellow. Other organic acids give a similar reaction. Mineral acids such as hydrochloric acid discharge the blue coloration leaving a colorless solution.

(*b*) *Ferric Chloride Test.*—Place 10 c.c. of *very dilute* ferric chloride

[1] Uffelmann's reagent is prepared by adding ferric chloride solution to a 1 per cent solution of carbolic acid until an amethyst-blue color is obtained.

in each of five tubes. To the first add 2 c.c. of 0.2 per cent hydrochloric acid, to the second 2 c.c. of 10 per cent alcohol, to the third 2 c.c. of 2 per cent sucrose, to the fourth 2 c.c. of lactic acid and to the fifth 2 c.c. of peptone solution.

It is evident from the results obtained that neither of the tests given above is satisfactory for the detection of lactic acid in the presence of other substances such as we find in the gastric contents.

A satisfactory deduction regarding the presence of lactic acid can only be made after extracting the gastric contents with ether, evaporating the ether extract to dryness, and dissolving the residue in water. This residue will not contain any of the contaminations which interfere with the simple tests as tried above, and therefore if either of the tests is now tried on the dissolved residue of the ether extract we may form an accurate conclusion regarding the presence of lactic acid.

(*c*) *Hopkins' Thiophene Reaction.*—Place about 5 c.c. of concentrated sulphuric acid in a test-tube and add one drop of a saturated solution of cupric sulphate.[1] Introduce a few drops of the solution to be tested, shake the tube well, and immerse it in the boiling water of a beaker-water-bath for one or two minutes. Now remove the tube, cool it under running water, add 2–3 drops of a dilute alcoholic solution[2] of thiophene, C_4H_4S, from a pipette, replace the tube in the beaker and carefully observe any color change which may occur. Lactic acid is indicated by the appearance of a bright *cherry-red* color which forms rapidly. This color may be made more or less permanent by cooling the tube as soon as the color is produced. Excess of thiophene produces a deep yellow or brown color with sulphuric acid. The test is not wholly specific though the author claims it to be more so than Uffelmann's reaction.

12. **Qualitative Analysis of Stomach Contents.**—Take 100 c.c. of stomach contents and analyze it according to the following scheme:

[1] This is added to catalyze the oxidation which follows.
[2] About 10–20 drops in 100 c.c. of 95 per cent alcohol.

Stomach Contents.

Filter and test the filtrate for free hydrochloric acid.

Filtrate I.
Divide into two parts.

Residue.
Discard after making a microscopical examination.

Filtrate II.
One-fifth portion.
Test for:
(*a*) Pepsin.
(*b*) Bile (see p. 151).
(*c*) Starch.
(*d*) Dextrin.

Filtrate III.
Four-fifths portion.
Neutralize carefully; any precipitate is acid metaprotein (acid albuminate). If a precipitate forms filter and divide the filtrate into *two* parts. If no precipitate forms divide the solution into *two* parts without filtering.

Filtrate IV.
Two-thirds portion.
Heat to boiling to remove coagulable proteins. If any precipitate forms filter it off; if there is no precipitate proceed directly with the tests.
Test for:
(*a*) Sugar. (Differentiate between the various sugars by the use of the scheme on page 50.)
(*b*) Proteoses.
(*c*) Peptones.

Filtrate V.
One-third portion.
Test for:
(*a*) Lactic acid.
(*b*) Gastric rennin.
(*c*) Salivary amylase.

CHAPTER VII.

FATS.

FATS occur very widely distributed in the plant and animal kingdoms, and constitute the third general class of food stuffs. In plant organisms they are to be found in the seeds, roots, and fruit while each individual tissue and organ of an animal organism contains more or less of the substance. In the animal organism fats are especially abundant in the bone marrow and adipose tissue. They contain the same elements as the carbohydrates, *i. e.*, carbon, hydrogen, and oxygen, but the oxygen is present in smaller percentage than in the carbodydrates and the hydrogen and oxygen are not present in the proportion to form water.

FIG. 35.—BEEF FAT. (*Long.*)

Chemically considered the fats are esters[1] of the tri-atomic alcohol, glycerol, and the mono-basic fatty acids. In the formation of these fats three molecules of water result. This water may arise in either of two ways. First, by the replacement of the H of each of the OH groups of glycerol by a fatty acid radical, giving the following formula in which R, R′ and R″ represent fatty acid radicals,

[1] An ester is an ethereal salt consisting of an organic radical united with the residue of an inorganic or organic acid.

$$\begin{array}{l} CH_2\cdot O\cdot R \\ | \\ CH\cdot O\cdot R' \\ | \\ CH_2\cdot O\cdot R''. \end{array}$$

Second, by the replacement of the H's of the carboxyl groups of the three fatty acid molecules by the glycerol radical, thus yielding the following type of formula in which R represents the glycerol radical,

$$\begin{array}{l} \quad\quad OOCH_{31}C_{15} \\ R\!-\!OOCH_{31}C_{15} \\ \quad\quad OOCH_{31}C_{15}. \end{array}$$

Of these two processes the second is the more logical procedure from the standpoint of the ionic theory. The three fatty acid radicals entering into the structure of a neutral fat may be the radicals of the same fatty acid or they may consist of the radicals of three different fatty acids.

By hydrolysis of a neutral fat, *i. e.*, by the addition to the molecule of those elements which are eliminated in the formation of the fat from glycerol and fatty acid, it may be resolved into its component parts, *i. e.*, glycerol and fatty acid. In the case of tripalmitin the following would be the reaction:

$$\underset{\text{Tri-palmitin.}}{C_3H_5(O\cdot C_{15}H_{31}CO)_3} + 3H_2O = \underset{\text{Glycerol.}}{C_3H_5(OH)_3} + 3\underset{\text{Palmitic acid.}}{(C_{15}H_{31}COOH)}.$$

This process is called *saponification* and may be produced by boiling with alkalis; by the action of steam under pressure; by long-continued contact with air and light; by the action of certain bacteria and by fat-splitting enzymes or lipases, *e. g.*, *pancreatic lipase* (see page 139). The cells forming the walls of the intestines evidently possess the peculiar property of synthesizing the glycerol and fatty acid thus formed so that after absorption these bodies appear in the blood not in their individual form but as neutral fats. This synthesis is similar to that enacted in the absorption of protein material where the peptones are synthesized into albumin in the act of absorption.

The principal animal fats with which we have to deal are *stearin*, *palmitin*, *olein*, and *butyrin*. Such less important forms as laurin and myristin may occur abundantly in plant organisms. The generally accepted system of nomenclature for these fats is to apply the prefix

"tri" in each case (*e. g.*, *tri*-palmitin) since three fatty acid radicals are contained in the neutral fat molecule.

Fats occur ordinarily as mixtures of several individual fats. For example, the fat found in animal tissues is a mixture of tri-olein, tri-palmitin and tri-stearin, the percentage of any one of these fats present depending upon the particular species of animal from whose tissue the fat was derived. Thus the ordinary mutton fat contains more tri-stearin and less tri-olein than the pork fat. Human fat contains from 67 per cent to 85 per cent of tri-olein and according to Benedict and Osterberg, upon analysis yields 76.08 per cent of carbon and 11.78 per cent of hydrogen.

Pure neutral fats are odorless, tasteless, and generally colorless. They are insoluble in the ordinary protein solvents such as water, salt solutions, and dilute acids and alkalis, but are very readily soluble in ether, benzene, chloroform, and *boiling* alcohol. The neutral fats are non-volatile substances possessing a *neutral* reaction. If allowed to remain in contact with the air for a sufficient length of time they become yellow in color, assume an *acid* reaction and are said to be *rancid*. The neutral fats may be crystallized, some of them with great facility. The crystalline forms of some of the more common fats are reproduced in Figs. 35, 36 and 37 on pages 128, 131 and 133. Each individual fat possesses a specific melting- or boiling-point (according to whether the body is solid or fluid in character) and this property of melting or boiling at a definite temperature may be used as a means of differentiation in the same way as the coagulation temperature (see page 109) is used for the differentiation of coagulable proteins. When shaken with water, or a solution of albumin, soap, or acacia, the liquid fats are finely divided and assume a condition known as an *emulsion*. The emulsion with water is transitory, while the emulsions with soap, acacia, or albumin, are permanent.

The fat ingested continues essentially unaltered until it reaches the intestines where it is acted upon by *pancreatic lipase* (*steapsin*) the fat-splitting enzyme of the pancreatic juice (see page 139), and glycerol and fatty acid are formed from a large portion of the fat. Part of the fatty acid thus formed is dissolved in the bile and absorbed while the remainder unites with the alkalis of the pancreatic juice and forms soluble soaps. These soaps may further act to produce an emulsion of the remaining fat and thus aid in its absorption. That bile is of assistance in the absorption of fat is indicated by the increase of fat in the feces when for any reason bile does not pass into the intestines. That fat is not absorbed unsplit in the form of an emulsion has recently

been redemonstrated by Whitehead[1] in a histological study of the absorption of fat stained with Sudan III.

The fat distributed throughout the animal body is formed partly from the ingested fat and partly from carbohydrates and the "carbon moiety" of protein material. The formation of *adipocere* and the occurrence of *fatty degeneration* are sometimes given as proofs of the formation of fat from protein. This is questioned by many investigators. Rather more satisfactory and direct proof of the formation of fat from protein material has been obtained by Hofmann in experi-

FIG. 36.—MUTTON FAT. (*Long.*)

mentation with *fly-maggots*. The normal content of fat in a number of maggots was determined and later the fat content of others which had developed in blood (84 per cent of the solid matter of blood plasma is protein material) was determined. The fat content was found to have increased 700 to 1100 per cent as a result of the diet of blood proteins. The celebrated experiments of Pettenkofer and Voit, however, have furnished what is, perhaps, the most substantial positive evidence of the formation of fat from protein. These investigators fed dogs large amounts of lean meat, daily, and through subsequent urinary and fecal examinations were enabled to account for *only part of the ingested carbon*, although obtaining a satisfactory nitrogen balance. The discrepancy in the carbon balance was explained upon the theory that the protein of the ingested meat had been split into a *nitrogenous* and a *non-nitrogenous* portion in the organism, and that the non-nitrogenous portion, the so-called "carbon moiety" of the protein, had been subsequently transformed into fat and deposited

[1] Whitehead: *American Journal of Physiology*, XXV, 1910. Proceedings p. 28.

as such in the tissues of the organism. Some investigators are not inclined to accept these data regarding the formation of fat from protein as conclusive.

The latest evidence in favor of the formation of fat from protein is furnished by the very recently reported experiments of Weinland. This investigator worked with the larvæ of *Calliphora*,[1] these larvæ being rubbed up in a mortar[2] with Witte's peptone and water to form a homogeneous mixture. After placing these mixtures at 38° C. for 24 hours the fat content was found to have increased, as much as 140 per cent in some instances. The active agency in this transformation of fat is the *larval tissue* since the tissues of both the dead and living larvæ possess the property. Data are given from control tests which show that the action of bacteria in this transformation of protein was excluded.

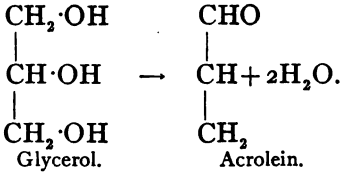
Experiments on Fats.

1. **Solubility.**—Test the solubility of olive oil in each of the ordinary solvents (see page 22) and in cold alcohol, hot alcohol, chloroform, ether, and carbon tetrachloride.

2. **Formation of a Transparent Spot on Paper.**—Place a drop of olive oil upon a piece of ordinary writing paper. Note the transparent appearance of the paper at the point of contact with the fat.

3. **Reaction.**—Try the reaction of *fresh* olive oil to litmus. Repeat the test with *rancid* olive oil. What is the reaction of a fresh fat and how does this reaction change upon allowing the fat to stand for some time?

4. **Formation of Acrolein.**—To a little olive oil in a mortar add some dry potassium bisulphate, $KHSO_4$, and rub up thoroughly. Transfer to a *dry* test-tube and cautiously heat. Note the irritating odor of *acrolein*. The glycerol of the fat has been dehydrolyzed and acrylic aldehyde or acrolein has been produced. This is the reaction which takes place:



[1] The ordinary "blow-fly."

[2] Intact larvæ were used in some experiments.

5. **Emulsification.**—(*a*) Shake up a drop of *neutral*[1] olive oil with a little water in a test-tube. The fat becomes finely divided, forming an emulsion. This is not a permanent emulsion since the fat separates and rises to the top upon standing.

(*b*) To 5 c.c. of water in a test-tube add 2 or 3 drops of 0.5 per cent Na_2CO_3. Introduce into this faintly alkaline solution a drop of *neutral* olive oil and shake. The emulsion while not permanent is not so transitory as in the case of water free from sodium carbonate.

(*c*) Repeat (*b*) using *rancid* olive oil. What sort of an emulsion do you get and why?

(*d*) Shake a drop of *neutral* olive oil with dilute albumin solution. What is the nature of this emulsion? Examine it under the microscope.

6. **Fat Crystals.**—Dissolve a small piece of lard in ether in a test-tube, add an equal volume of alcohol and allow the alcohol-

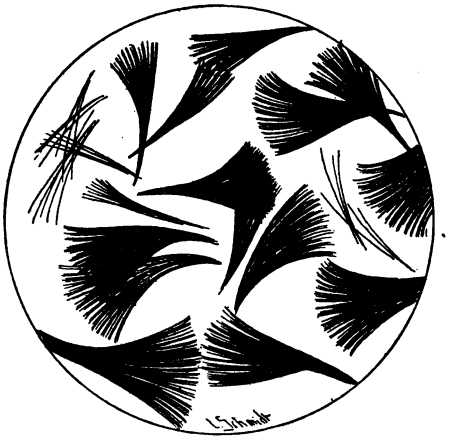


FIG. 37.—PORK FAT.

ether mixture to evaporate spontaneously. Examine the crystals under the microscope and compare them with those reproduced in Figs. 35, 36 and 37, on pages 128, 131 and 133.

7. **Saponification of Bayberry Tallow.**[2]—Fill a large casserole two-thirds full of water rendered strongly alkaline with *solid* potassium hydroxide (a stick one inch in length). Add about 10 grams of bayberry tallow and boil, keeping the volume constant by adding

[1] Neutral olive oil may be prepared by shaking ordinary olive oil with a 10 per cent solution of sodium carbonate. This mixture should then be extracted with ether and the ether removed by evaporation. The residue is *neutral* olive oil.

[2] Bayberry tallow is derived from the fatty covering of the berries of the *wax myrtle*. It is therefore frequently called "myrtle wax" or "bayberry wax."

water as needed. When saponification is complete[1] remove 25 c.c. of the soap solution for use in Experiment 8 and add concentrated hydrochloric acid slowly to the remainder until no further precipitate is produced.[2] Cool the solution and the precipitate of free fatty acid will rise to the surface and form a cake. In this instance the fatty acid is principally *palmitic* acid. Remove the cake, break it into small pieces, wash it with water by decantation and transfer to a small beaker by means of 95 per cent alcohol. Heat on a water-bath until the palmitic acid is dissolved, then filter through a dry filter

FIG. 38.—PALMITIC ACID.

paper and allow the filtrate to cool slowly in order to obtain satisfactory crystals. Write the reactions which have taken place in this experiment.

When the palmitic acid has completely crystallized filter off the alcohol, dry the crystals between filter papers and try the tests given in Experiment 9, below.

8. **Salting-out Experiment.**—To 25 c.c. of soap solution, prepared as described above, add *solid* sodium chloride to the point of saturation, with continual stirring. A menstruum is thus formed in which the soap is insoluble. This salting-out process is entirely analogous to the salting-out of proteins (see page 94).

9. **Palmitic Acid.**—(*a*) Examine the crystals under the microscope and compare them with those shown in Fig. 38, above.

[1] Place 2 or 3 drops in a test-tube full of water. If saponification is complete the products will remain in solution and no oil will separate.

[2] Under some conditions a purer product is obtained if the soap solution is cooled *before* precipitating the fatty acid.

(*b*) *Solubility.*—Try the solubility of palmitic acid in the same solvents as used on fats (see page 132).

(*c*) *Melting-point.*—Determine the melting-point of palmitic acid by one of the methods given on page 136.

(*d*) *Formation of Transparent Spot on Paper.*—Melt a little of the fatty acid and allow a drop to fall upon a piece of ordinary writing paper. How does this compare with the action of a fat under similar circumstances?

(*e*) *Acrolein Test.*—Apply the test as given under 4, page 132. Explain the result.

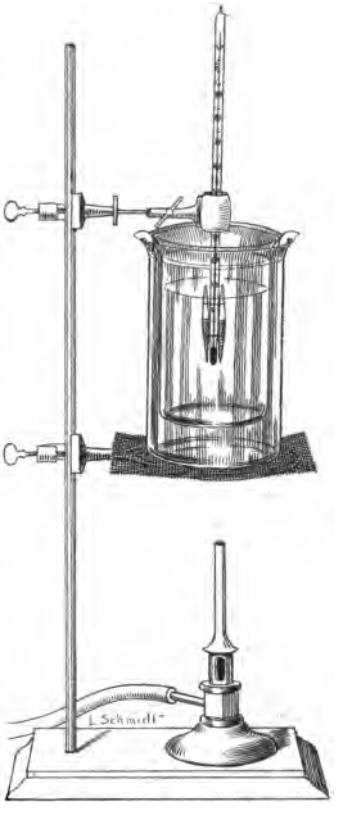


FIG. 39.—MELTING-POINT APPARATUS.

10. **Saponification of Lard.**—To 25 grams of lard in a flask add 75 c.c. of alcoholic-potash solution and warm upon a water-bath until saponification is complete. (This point is indicated by the complete solubility of a drop of the solution when allowed to fall into a little water.) Now transfer the solution from the flask to an evaporating dish containing about 100 c.c. of water and heat on a water-bath until all the alcohol has been driven off. Precipitate the fatty acid with hydrochloric acid and cool the solution. Remove the fatty acid which rises to the surface, neutralize the solution with sodium carbonate and evaporate to dryness. Extract the residue with alcohol, remove the alcohol by evaporation upon a water-bath and on the residue of glycerol thus obtained make the tests as given below.

11. **Glycerol.** (*a*) *Taste.*—What is the taste of glycerol?

(*b*) *Solubility.*—Try the solubility of glycerol in water, alcohol and ether.

(*c*) *Acrolein Test.*—Repeat the test as given under 4, page 132.

(*d*) *Borax Fusion Test.*—Fuse a little glycerol on a platinum wire with some powdered borax and note the characteristic green flame. This color is due to the glycerol ester of boric acid.

(*e*) *Fehling's Test.*—How does this result compare with the results on the sugars?

(*f*) *Solution of* $Cu(OH)_2$.—Form a little cupric hydroxide by mixing cupric sulphate and potassium hydroxide. Add a little glycerol to this suspended precipitate and note what occurs.

12. **Melting-Point of Fat.** *First Method.*—Insert one of the melting-point tubes, furnished by the instructor, into the liquid fat and draw up the fat until the bulb of the tube is about one-half full of the material. Then fuse one end of the tube in the flame of a bunsen burner and fasten the tube to a thermometer by means of a rubber band in such a manner that the bottom of the fat column is on a level with the bulb of the thermometer (Fig. 39, page 135). Fill a beaker of medium size about two-thirds full of water and place it within a second larger beaker which also contains water, the two vessels being separated by pieces of cork. Immerse the bulb of the thermometer and the attached tube in such a way that the bulb is about midway between the upper and the lower surfaces of the water of the inner beaker. The upper end of the tube being open it must extend above the surface of the surrounding water. Apply gentle heat, stir the water, and note the temperature at which the fat first begins to melt. This point is indicated by the initial transparency. For ordinary fats, raise the temperature very cautiously from 30° C. To determine the *congealing-point* remove the flame and note the temperature at which the fat begins to solidify. Record the melting- and congealing-points of the various fats submitted by the instructor.

Second Method.—Fill a small evaporating dish about one-half full of mercury and place it on a water-bath. Put a small drop of the fat under examination on an ordinary coverglass and place this upon the surface of the mercury. Raise the temperature of the water-bath slowly and by means of a thermometer whose bulb is immersed in the mercury, note the melting-point of the fat. Determine the congealing-point by removing the flame and leaving the fat drop and coverglass in position upon the mercury. How do the melting-points as determined by this method compare with those as determined by the first method? Which method is the more accurate, and why?

CHAPTER VIII.

PANCREATIC DIGESTION.

As soon as the food mixture leaves the stomach it comes into intimate contact with the bile and the pancreatic juice. Since these fluids are alkaline in reaction there can obviously be no further peptic activity after they have become intimately mixed with the chyme and have neutralized the acidity previously imparted to it by the hydrochloric acid of the gastric juice. The pancreatic juice reaches the intestine through the duct of Wirsung which opens into the intestine near the pylorus.

Normally the secretion of pancreatic juice is brought about by the stimulation produced by the acid chyme as it enters the duodenum. This secretion is probably not due to a nervous reflex as was believed by Pawlow but rather, as Bayliss and Starling have shown, is dependent upon the presence, in the epithelial cells of the duodenum and jejunum of a body known as *prosecretin*. This body is changed into *secretin*[1] through the hydrolytic action of the acid present in the chyme. The secretin is then absorbed by the blood, passes to the pancreas and stimulates the pancreatic cells, causing a flow of pancreatic juice. The quantity of juice secreted under these conditions is proportional to the amount of secretin present. The activity of secretin solutions is not diminished by boiling, hence the body does not react like an enzyme. Further study of the body may show it to be a definite chemical individual of relatively low molecular weight. It has not been possible thus far to obtain secretin from any tissues except the mucous membrane of the duodenum and jejunum.

The juice as obtained from a permanent fistula differs greatly in its properties from the juice as obtained from a temporary fistula, and neither form of fluid possesses the properties of the normal fluid. Pancreatic juice collected by Glaessner from a natural fistula has been found to be a colorless, clear, strongly alkaline fluid which foams readily. It is further characterized by containing albumin, globulin, proteose, and peptone; nucleoprotein is also present in traces.[2] The average daily secretion of pancreatic juice is 650 c.c. and its specific

[1] Secretin belongs to the class of substances called *hormones* or chemical messengers.
[2] Glaessner: *Zeitschrift für physiologische Chemie*, 1904, 40, p. 476.

gravity is 1.008. The fluid contains 1.3 per cent of solid matter and the freezing-point is —0.47° C. The normal pancreatic secretion contains at least four distinct enzymes. They are *trypsin*, a proteolytic enzyme; *pancreatic amylase* (amylopsin), an amylolytic enzyme; *pancreatic lipase* (steapsin), a fat-splitting enzyme; and *pancreatic rennin*, a milk-coagulating enzyme. *Lactase*, the lactose-splitting enzyme, is also present at certain times.

The most important of the four enzymes of the pancreatic juice is the proteolytic enzyme trypsin. This enzyme resembles pepsin in so far as each has the power of breaking down protein material, but the trypsin has much greater digestive power and is able to cause a more complete decomposition of the complex protein molecule. In the process of normal digestion the protein constituents of the diet are for the most part transformed into proteoses (albumoses) and peptones before coming in contact with the enzyme trypsin. This is not absolutely essential however, since trypsin possesses digestive activity sufficient to transform unaltered native proteins and to produce from their complex molecules comparatively simple fragments. Among the products of tryptic digestion are *proteoses*, *peptones*, *peptides*, *leucine*, *tyrosine*, *aspartic acid*, *glutamic acid*, *alanine*, *phenylalanine*, *glycocoll*, *cystine*, *serine*, *valine*, *proline*, *oxyproline*, *isoleucine*, *arginine*, *lysine*, *histidine*, and *tryptophane*. (The crystalline forms of many of these products are reproduced in Chapter IV.) Trypsin does not occur preformed in the gland, but exists there as a zymogen called *trypsinogen* which bears the same relation to trypsin that pepsinogen does to pepsin. Trypsin has never been obtained in a pure form and therefore very little can be stated definitely as to its nature. The enzyme is the most active in alkaline solution but is also active in neutral or slightly acid solutions. Trypsin is destroyed by mineral acids and may also be destroyed by comparatively weak alkali (2 per cent sodium carbonate) if left in contact for a sufficiently long time. Trypsinogen, on the other hand, is more resistant to the action of alkalis. In pancreatic digestion the protein does not swell as is the case in gastric digestion, but becomes more or less "honeycombed" and it finally disintegrates.

The pancreatic juice which is collected by means of a fistula possesses practically no power to digest protein matter. A body called *enterokinase* occurs in the intestinal juice and has the power of converting trypsinogen into trypsin. This process is known as the "activation" of trypsinogen and through it a juice which is incapable of digesting protein may be made active. Enterokinase is not always

present in the intestinal juice since it is secreted only after the pancreatic juice reaches the intestine. It resembles the enzymes in that its activity is destroyed by heat, but differs materially from this class of bodies in that a certain quantity is capable of activating only a definite quantity of trypsinogen. It is, however, generally classified as an enzyme. Enterokinase has been detected in the higher animals, and a kinase possessing similar properties has been shown to be present in bacteria, fungi, impure fibrin, lymph glands, and snake-venom. The activation of trypsinogen into trypsin may be brought about in the gland as well as in the intestine of the living organism (Mendel and Rettger). The manner of the activation in the gland and the nature of the body causing it are unknown at present.

Delezenne claims that trypsinogen may be activated by soluble *calcium salts*. He reports experiments which indicate that proteolytically inactive pancreatic juice, obtained directly from the duct, when treated with salts of this character, assumes the property of digesting protein material. This process by which the trypsinogen is activated through the instrumentality of calcium salts is very rapid and is designated by Delezenne as an "explosion." The recent suggestion of Mays that there may possibly be several precursors of trypsin one of which is activated by enterokinase and the others by other agents, is of interest in this connection.

Pancreatic amylase (*amylopsin*), the second of the pancreatic enzymes, is an amylolytic enzyme which possesses somewhat greater digestive power than the salivary amylase (ptyalin) of the saliva. As its name implies, its activity is confined to the starches, and the products of its amylolytic action are dextrins and sugars. The sugars are principally iso-maltose and maltose and these by the further action of an inverting enzyme are partly transformed into dextrose.

It is possible that the saliva as a digestive fluid is not absolutely essential. The salivary amylase (ptyalin) is destroyed by the hydrochloric acid of the gastric juice and is therefore inactive when the chyme reaches the intestine. Should undigested starch be present at this point however, it would be quickly transformed by the active pancreatic amylase. This enzyme is not present in the pancreatic juice of infants during the first few weeks of life, thus showing very clearly that a starchy diet is not normal for this period.

It has been claimed that pancreatic amylase has a slight digestive action upon *unboiled* starch.

The third enzyme of the pancreatic juice is called *pancreatic lipase* (*steapsin*) and is a fat-splitting enzyme. It has the power of splitting

the neutral fats of the food by hydrolysis, into fatty acid and glycerol. A typical reaction would be as follows:

$$\underset{\text{Tri-palmitin.}}{C_3H_5(O\cdot C_{15}H_{31}CO)_3} + 3H_2O = 3(\underset{\text{Palmitic acid.}}{C_{15}H_{31}COOH}) + \underset{\text{Glycerol.}}{C_3H_5(OH)_3}.$$

Recent researches make it probable that fats undergo saponification to a certain extent prior to their absorption. The fatty acids formed, in part unite with the alkalis of the pancreatic juice and intestinal secretion to form soluble soaps; in part they are doubtless absorbed dissolved in the bile. Some observers believe that the fats may also be absorbed in emulsion—a condition promoted by the presence of the soluble soaps. After absorption the fatty acids are re-synthesized to form neutral fats with glycerol.

Pancreatic lipase is very unstable and is easily rendered inert by the action of acid. For this reason it is not possible to prepare an extract having a satisfactory fat-splitting power from a pancreas which has been removed from the organism for a sufficiently long time to have become acid in reaction.

The fourth enzyme of the pancreatic juice is called *pancreatic rennin*. It is a milk-coagulating enzyme whose action is very similar to that of the enzyme *gastric rennin* found in the gastric juice. It is supposed to show its greatest activity at a temperature varying from 60° to 65° C.

The enzymes of the intestinal juice are of great importance to the animal organism. These enzymes include *erepsin* (erepsase), *sucrase, maltase, lactase*, and *enterokinase*.

Erepsin is a proteolytic enzyme which has the property of acting upon the proteoses and peptones which are formed through the action of trypsin and further splitting them into *amino acids*. Erepsin has no power of digesting any native proteins except caseinogen, histones, and protamines. It possesses its greatest activity in an alkaline solution although it is slightly active in acid solution. An extract of the intestinal erepsin may be prepared by treating the finely divided intestine of a *cat, dog*, or *pig* with toluene- or chloroform-water and permitting the mixture to stand with occasional shaking for 24–72 hours.[1] Enzymes similar to erepsin occur in various tissues of the organism.

The three invertases *sucrase, maltase*, and *lactase* are also important enzymes of the intestinal mucosa. The sucrase acts upon sucrose

[1] See p. 12.

and inverts it with the formation of *invert sugar* (dextrose and lævulose). Some investigators claim that sucrase is also present in saliva and gastric juice. It probably does not exist normally in either of these digestive juices, however, and if found owes its presence to the excretory processes of certain bacteria. Sucrases may also be obtained from several vegetable sources. For investigational purposes it is ordinarily obtained from yeast (see p. 11). It exhibits its greatest activity in the presence of a slight acidity but if the acidity be increased to any extent the reaction is inhibited.

Lactase is an enzyme which inverts lactose with the consequent formation of dextrose and galactose. Its action is entirely analogous, in type, to that of sucrase. It has apparently been proven that lactase occurs in the intestinal mucosa of the young of all animals which suckle their offspring.[1] It may also occur in the intestinal mucosa of certain adult animals if such animals be maintained upon a ration containing more or less lactose. Fischer and Armstrong have demonstrated the reversible action[2] of lactase.

For discussions of *maltase* and *enterokinase* see pages 54 and 138 respectively.

PREPARATION OF AN ARTIFICIAL PANCREATIC JUICE.[3]

After removing the fat from the pancreas of a pig or sheep, finely divide the organ by means of scissors and grind it in a mortar. If convenient, the use of an ordinary meat chopper is a very satisfactory means of preparing the pancreas.

When finely divided as above the pancreas should be placed in a 500 c.c. flask, about 150 c.c. of 30 per cent alcohol added and the flask and contents shaken frequently for twenty-four hours. (What is the reaction of this alcoholic extract at the end of this period, and why?) Strain the alcoholic extract through cheese cloth, filter, nearly neutralize with potassium hydroxide solution and then exactly neutralize it with 0.5 per cent sodium carbonate.

Products of Tryptic Digestion.

Take about 200 grams of lean beef which has been freed from fat and finely ground and place it in a large-sized beaker. Introduce equal volumes of the pancreatic extract prepared as above and 0.5

[1] Mendel and Mitchell: *American Journal of Physiology*, 1907, XX, p. 81.
[2] See p. 6.
[3] For other methods of preparation see Karl Mays: *Zeitschrift für physiologische Chemie*, 1903, XXXVIII, p. 428.

per cent sodium carbonate, add 5 c.c. of an alcoholic solution of thymol to prevent putrefaction, and place the beaker in an incubator at 40° C. Stir the contents of the beaker frequently and add more thymol if it becomes necessary. Allow digestion to proceed for from 2 to 5 days and then separate the products formed as follows: Strain off the undissolved residue through cheese cloth, nearly neutralize the solution with dilute hydrochloric acid and then exactly neutralize it with 0.2 per cent hydrochloric acid. A precipitate at this point would indicate *alkali metaprotein* (alkali albuminate). Filter off any precipitate and divide the filtrate into two parts, a one-fourth and a three-fourth portion.

Transfer the one-fourth portion to an evaporating dish and make the separation of *proteoses* and *peptones* as well as the final tests upon these bodies according to the directions given on page 110.

Place about 5 c.c. of the three-fourth portion in a test-tube and add about 1 c.c. of bromine water. A violet coloration indicates the presence of *tryptophane* (see page 72). Concentrate[1] the remainder of the three-fourth portion to a thin syrup and make the separation of *leucine* and *tyrosine* according to the directions given on page 80.

GENERAL EXPERIMENTS ON PANCREATIC DIGESTION.

Experiments on Trypsin.

1. **The Most Favorable Reaction for Tryptic Digestion.**— Prepare seven tubes as follows:

(*a*) 2–3 c.c. of *neutral* pancreatic extract + 2–3 c.c. of water.

(*b*) 2–3 c.c. of *neutral* pancreatic extract + 2–3 c.c. of 1 per cent sodium carbonate.

(*c*) 2–3 c.c. of *neutral* pancreatic extract + 2–3 c.c. of 0.5 per cent sodium carbonate.

(*d*) 2–3 c.c. of *neutral* pancreatic extract + 2–3 c.c. of 0.2 per cent hydrochloric acid.

(*e*) 2–3 c.c. of *neutral* pancreatic extract + 2–3 c.c. of 0.2 per cent *combined* hydrochloric acid.

(*f*) 2–3 c.c. of *neutral* pancreatic extract + 2–3 c.c. of 0.4 per cent boric acid.

(*g*) 2–3 c.c. of *neutral* pancreatic extract + 2–3 c.c. of 0.4 per cent acetic acid.

[1] If the solution is alkaline in reaction, while it is being concentrated, the amino acids will be broken down and ammonia will be liberated.

Add a small piece of fibrin to the contents of each tube and keep them at 40° C. noting the progress of digestion. In which tube do we find the most satisfactory digestion, and why? How do the indications of the digestion of fibrin by trypsin differ from the indications of the digestion of fibrin by pepsin?

2. **The Most Favorable Temperature.**—(For this and the following series of experiments under tryptic digestion use the *neutral* extract plus an *equal volume* of 0.5 per cent sodium carbonate.) In each of four tubes place 5 c.c. of *alkaline* pancreatic extract. Immerse one tube in cold water from the faucet, keep a second at room temperature and place a third on the water-bath at 40° C. Boil the contents of the fourth for a few moments, then cool and also keep it at 40° C. Into each tube introduce a small piece of fibrin and note the progress of digestion. In which tube does the most rapid digestion occur? What is the reason?

3. **Influence of Metallic Salts, Etc.**—Prepare a series of tubes and into each tube place 6 volumes of water, 3 volumes of alkaline pancreatic extract and 1 volume of one of the chemicals listed in Experiment 18 under Salivary Digestion, page 58.

Introduce a small piece of fibrin into each of the tubes and keep them at 40° C. for one-half hour. Shake the tubes frequently. In which tubes do we get the least digestion?

4. **Influence of Bile.**—Prepare five tubes as follows:

(*a*) Five c.c. of pancreatic extract + 1/2–1 c.c. of bile.

(*b*) Five c.c. of pancreatic extract + 1–2 c.c. of bile.

(*c*) Five c.c. of pancreatic extract + 2–3 c.c. of bile.

(*d*) Five c.c. of pancreatic extract + 5 c.c. of bile.

(*e*) Five c.c. of pancreatic extract.

Introduce into each tube a small piece of fibrin and keep them at 40° C. Shake the tubes frequently and note the progress of digestion. Does the presence of bile retard tryptic digestion? How do these results agree with those obtained under gastric digestion?

Experiments on Pancreatic Amylase.

1. **The Most Favorable Reaction.**—Prepare seven tubes as follows:

(*a*) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of water.

(*b*) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 1 per cent sodium carbonate.

(*c*) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.5 per cent sodium carbonate.

(*d*) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.2 per cent hydrochloric acid.

(*e*) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.2 per cent *combined* hydrochloric acid.

(*f*) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.4 per cent boric acid.

(*g*) One c.c. of *neutral* pancreatic extract + 1 c.c. of starch paste + 2 c.c. of 0.4 per cent acetic acid.

Shake each tube thoroughly and place them on the water-bath at 40° C. At the end of a half-hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Fehling's test. Where do you find the most satisfactory digestion? How do the results compare with those obtained from the similar series under Trypsin, page 142?

2. **The Most Favorable Temperature.**—(For this and the following series of experiments upon pancreatic amylase use the ***neutral*** extract plus an equal volume of 0.5 per cent sodium carbonate.) In each of four tubes place 2–3 c.c. of *alkaline* pancreatic extract. Immerse one tube in cold water from the faucet, keep a second at room temperature, and place a third on the water-bath at 40° C. Boil the contents of the fourth for a few moments, then cool and also keep it at 40° C. Into each tube introduce 2–3 c.c. of starch paste and note the progress of digestion. At the end of one-half hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Fehling's test. In which tube do you find the most satisfactory digestion? How does this result compare with the result obtained in the similar series of experiments under Trypsin (see page 143)?

3. **Influence of Metallic Salts, etc.**—Prepare a series of tubes and into each place 3 volumes of water, 3 volumes of *alkaline* pancreatic extract, 1 volume of one of the chemicals listed in Experiment 18 under Salivary Digestion, page 58, and 3 volumes of starch paste. Be sure to introduce the *starch paste* into the tube *last.* Why? Shake the tubes well and place them on the water-bath at 40° C. At the end of a half-hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Fehling's test. What are your conclusions?

4. **Influence of Bile.**—Prepare five tubes as follows:

(*a*) 2–3 c.c. of pancreatic extract + 2–3 c.c. of starch paste + 1/2–1 c.c. of bile.

(*b*) 2–3 c.c. of pancreatic extract + 2–3 c.c. of starch paste + 1–2 c.c. of bile.

(*c*) 2–3 c.c. of pancreatic extract + 2–3 c.c. of starch paste + 2–3 c.c. of bile.

(*d*) 2–3 c.c. of pancreatic extract + 2–3 c.c. of starch paste + 5 c.c. of bile.

(*e*) 2–3 c.c. of pancreatic extract + 2–3 c.c. of starch paste.

Shake the tubes thoroughly and place them on the water-bath at 40° C. Note the progress of digestion frequently and at the end of a half-hour divide the contents of each tube into two parts and test one part by the iodine test and the other part by Fehling's test. What are your conclusions regarding the influence of bile upon the action of pancreatic amylase?

5. **Digestion of Dry Starch.**—To a little *dry* starch in a test-tube add about 5 c.c. of pancreatic extract and place the tube on the water-bath at 40° C. At the end of a half-hour filter and test separate portions of the filtrate by the iodine and Fehling tests. What do you conclude regarding the action of pancreatic amylase upon dry starch? Compare this result with that obtained in the similar experiment under Salivary Digestion (page 57).

6. **Digestion of Inulin.**—To 5 c.c. of inulin solution in a test-tube add 10 drops of pancreatic extract and place the tube on the water-bath at 40° C. After one-half hour test the solution by Fehling's test.[1] Is any reducing substance present? What do you conclude regarding the digestion of inulin by pancreatic amylase?

Experiments on Pancreatic Lipase.

1. **"Litmus-milk" Test.**—Into each of two test-tubes introduce 10 c.c. of milk and a small amount of litmus powder. To the contents of one tube add 3 c.c. of *neutral* pancreatic extract and to the contents of the other tube add 3 c.c. of water or of *boiled* neutral pancreatic extract. Keep the tubes at 40° C. and note any changes which may occur. What is the result and how do you explain it?

2. **Ethyl Butyrate Test.**—Into each of two test-tubes introduce 4 c.c. of water, 2 c.c. of ethyl butyrate, $C_3H_7COO.C_2H_5$, and a small amount of litmus powder. To the contents of one tube add 4 c.c. of *neutral* pancreatic extract and to the contents of the other tube add 4 c.c. of water of *boiled* neutral pancreatic extract. Keep the tubes at

[1] If the inulin solution gives a reduction before being acted upon by the pancreatic juice, it will be necessary to determine the extent of the original reduction by means of a "check" test (see page 47).

40° C. and observe any changes which may occur. What is the result and how do you explain it? Write the equation for the reaction which has taken place.

Experiments on Pancreatic Rennin.

Prepare four test-tubes as follows:

(*a*) Five c.c. of milk + 10 drops of *neutral* pancreatic extract.

(*b*) Five c.c. of milk + 20 drops of *neutral* pancreatic extract.

(*c*) Five c.c. of milk + 10 drops of *alkaline* pancreatic extract.

(*d*) Five c.c. of milk + 20 drops of *alkaline* pancreatic extract.

Place the tubes at 60°–65° C. for a half hour *without shaking*. Note the formation of a clot.[1] How does the action of pancreatic rennin compare with the action of the gastric rennin?

[1] This reaction will not always succeed, owing to conditions which are not well understood.

CHAPTER IX.

BILE.

THE bile is secreted continuously by the liver and passes into the intestine through the common bile duct which opens near the pylorus. Bile is *not* secreted continuously *into the intestine.* In a fasting animal no bile enters the intestine, but when food is taken the bile begins to flow; the length of time elapsing between the ingestion of the food and the secretion of the bile as well as the qualitative and quantitative characteristics of the secretion depending upon the nature of the food ingested. Fats, the extractives of meat and the protein end-products of gastric digestion (proteoses and peptones), cause a copious secretion of bile, whereas such substances as water, acids and boiled starch paste fail to do so. In general a rich protein diet is supposed to increase the amount of bile secreted, whereas a carbohydrate diet would cause a much less decided increase and might even tend to decrease the amount. It has been demonstrated by Bayliss and Starling that the secretion of bile is under the control of the same mechanism that regulates the flow of pancreatic juice (see p. 137). In other words, the hydrochloric acid of the chyme, as it enters the duodenum transforms prosecretin into secretin and this in turn enters the circulation, is carried to the liver, and stimulates the bile-forming mechanism to increased activity.

We may look upon the bile as an *excretion* as well as a *secretion.* In the fulfillment of its excretory function it passes such bodies as lecithin, metallic substances, cholesterol, and the decomposition products of hæmoglobin into the intestine and in this way aids in removing them from the organism. The bile assists materially in the absorption of fats from the intestine by its solvent action on the fatty acids formed by the action of the pancreatic juice.

The bile is a ropy, viscid substance which is alkaline in reaction to litmus,[1] and ordinarily possesses a decidedly bitter taste. It varies in color in the different animals, the principal variations being yellow, brown, and green. Fresh human bile from the living organism ordinarily has a green or golden-yellow color. Postmortem bile is variable in color. It is very difficult to determine accurately the amount of normal bile secreted during any given period. For an adult man it

[1] It does not contain any *free hydroxyl ions*, however.

has been variously estimated at from 500 c.c. to 1100 c.c. for twenty-four hours. The specific gravity of the bile varies between 1.010 and 1.040, and the freezing-point is about —0.56° C. As secreted by the liver, the bile is a clear, limpid fluid which contains a relatively low content of solid matter. Such bile would have a specific gravity of approximately 1.010. After it reaches the gall-bladder, however, it becomes mixed with mucous material from the walls of the gall-bladder, and this process coupled with the continuous absorption of water from the bile has a tendency to concentrate the secretion. Therefore the bile as we find it in the gall-bladder, ordinarily possesses a higher specific gravity than that of the freshly secreted fluid. The specific gravity under these conditions may run as high as 1.040.

The principal constituents of the bile are the *salts of the bile acids*, *bile pigments*, *neutral fats*, *lecithin*, *phosphatides*, and *cholesterol*, besides the salts of *iron*, *copper*, *calcium*, and *magnesium*. *Zinc* has also frequently been found in traces.

The bile acids, which are elaborated exclusively by the hepatic cells, may be divided into two groups, the *glycocholic acid* group and the *taurocholic acid* group. In human bile glycocholic acid predominates, while taurocholic acid is the more abundant in the bile of carnivora. The bile acids are conjugate *amino-acids*, the glycocholic acid yielding *glycocoll*,

$$\begin{array}{l} CH_2 \cdot NH_2 \\ | \\ COOH, \end{array}$$

and *cholic acid* upon decomposition, whereas taurocholic acid gives rise to *taurine*,

$$\begin{array}{l} CH_2 \cdot NH_2 \\ | \\ CH_2 \cdot SO_2 \cdot OH, \end{array}$$

and *cholic acid* under like conditions. Glycocholic acid contains some nitrogen but no sulphur, whereas taurocholic acid contains both these elements. The sulphur of the taurocholic acid is present in the taurine (amino-ethyl-sulphonic-acid), of which it is a characteristic constituent. There are several varieties of cholic acid and therefore we have several forms of glycocholic and taurocholic acids, the variation in constitution depending upon the nature of the cholic acid which enters into the combination. The bile acids are present in the bile as salts of one of the alkalis, generally sodium. The sodium glycocholate and sodium taurocholate may be isolated in crystalline form,

either as balls or rosettes of fine needles or in the form of prisms having ordinarily four or six sides (Fig. 40, below). The salts of the bile acids are dextro-rotatory. Among other properties these salts have the power of holding the cholesterol and lecithin of the bile in solution.

Hammarsten has demonstrated a third group of bile acids in the bile of the shark. This same group very probably occurs in certain other animals also. These acids are very rich in sulphur and resemble ethereal sulphuric acids inasmuch as upon treatment with boiling hydrochloric acid they yield sulphuric acid.

FIG. 40.—BILE SALTS.

The bile pigments are important and interesting biliary constituents. The following have been isolated: *bilirubin*, *biliverdin*, *bilifuscin*, *biliprasin*, *bilihumin*, *bilicyanin*, *choleprasin*, and *choletelin*. Of these, bilirubin and biliverdin are the most important and predominate in normal bile. The colors possessed by the various varieties of normal bile are due almost entirely to these two pigments, the biliverdin being the predominant pigment in greenish bile and the bilirubin being the principal pigment in lighter colored bile. The pigments, other than the two just mentioned, have been found almost exclusively in biliary calculi or in altered bile obtained at post-mortem examinations.

Bilirubin, which is perhaps the most important of the bile pigments, is apparently derived from the blood pigment, the iron freed in the process being held in the liver. Bilirubin has the same percentage composition as hæmatoporphyrin, which may be produced from hæmatin. It is a specific product of the liver cells, but may also be formed in other parts of the body. The pigment may be isolated in the form of a reddish-yellow powder or may be obtained in part, in the form of

reddish-yellow rhombic plates (Fig. 41, below) upon the spontaneous evaporation of its chloroform solution. The crystalline form of bilirubin is practically the same as that of hæmatoidin. It is easily soluble in chloroform, somewhat less soluble in alcohol and only slightly soluble in ether and benzene. Bilirubin has the power of combining with certain metals, particularly calcium, to form combinations which are no longer soluble in the solvents of the unaltered pigment. Upon long standing in contact with the air, the reddish-yellow bilirubin is oxidized with the formation of the green biliverdin. Bilirubin occurs in animal fluids as soluble bilirubin-alkali.

FIG. 41.—BILIRUBIN (HÆMATOIDIN). (*Ogden.*)

Solutions of bilirubin exhibit no absorption-bands. If an ammoniacal solution of bilirubin-alkali in water is treated with a solution of zinc chloride, however, it shows bands similar to those of bilicyanin (Absorption Spectra, Plate II), the two bands between C and D being rather well defined.

Biliverdin is particularly abundant in the bile of herbivora. It is soluble in alcohol and glacial acetic acid and insoluble in water, chloroform, and ether. Biliverdin is formed from bilirubin upon oxidation. It is an amorphous substance, and in this differs from bilirubin which may be at least partly crystallized under proper conditions. Biliverdin may be obtained in the form of a green powder. In common with bilirubin, it may be converted into hydrobilirubin by nascent hydrogen.

The neutral solution of bilicyanin or cholecyanin is bluish-green or steel-blue and possesses a blue fluorescence, the alkaline solution is green with no appreciable fluorescence and the strongly acid solution is violet-blue. The alkaline solution exhibits three absorption-bands, the first a dark, well-defined band between C and D, somewhat nearer C; the second a less sharply-defined band extend-

ing across D and the third a rather faint band between E and F, near E (Absorption Spectra, Plate II). The strongly acid solution exhibits two absorption bands, both lying between C and E and separated by a narrow space near D. A third band, exceedingly faint, may ordinarily be seen between b and F.

Biliary calculi, otherwise designated as *biliary concretions* or *gall stones*, are frequently formed in the gall-bladder. These deposits may be divided into three classes, *cholesterol calculi*, *pigment calculi*, and calculi made up almost entirely of *inorganic material*. This last class of calculus is formed principally of the carbonate and phosphate of calcium and is rarely found in man although quite common to cattle. The pigment calculus is also found in cattle, but is more common to man than the inorganic calculus. This pigment calculus ordinarily consists principally of bilirubin in combination with calcium; biliverdin is sometimes found in small amount. The cholesterol calculus is the one found most frequently in man. These may be formed almost entirely of cholesterol, in which event the color of the calculus is very light, or they may contain more or less pigment and inorganic matter mixed with the cholesterol, which tends to give us calculi of various colors.

For discussion of cholesterol see page 246.

Experiments on Bile.

1. **Reaction.**—Test the reaction of fresh ox bile to litmus.

2. **Nucleoprotein.**—Acidify a small amount of bile with dilute acetic acid. A precipitate of nucleoprotein forms.

3. **Inorganic Constituents.**—Test for chlorides, sulphates, and phosphates (see page 56).

4. **Tests for Bile Pigments.** (*a*) *Gmelin's Test.*—To about 5 c.c. of *concentrated* nitric acid in a test-tube add 2–3 c.c. of diluted bile *carefully* so that the two fluids do not mix. At the point of contact note the various colored rings, green, blue, violet, red and reddish-yellow. Repeat this test with different dilutions of bile and observe its delicacy.

(*b*) *Rosenbach's Modification of Gmelin's Test.*—Filter 5 c.c. of diluted bile through a small filter paper. Introduce a drop of *concentrated* nitric acid into the cone of the paper and note the succession of colors as given in Gmelin's test.

(*c*) *Nakayama's Reaction.*—To 5 c.c. of diluted bile in a test-tube add an equal volume of a 10 per cent solution of barium chloride, centrifugate the mixture, pour off the supernatant fluid, and heat

the precipitate with 2 c.c. of Nakayama's reagent.[1] In the presence of bile pigments the solution assumes a blue or green color.

(*d*) *Huppert's Reaction.*—Thoroughly shake equal volumes of undiluted bile and milk of lime in a test-tube. The pigments unite with the calcium and are precipitated. Filter off the precipitate, wash it with water, and transfer to a small beaker. Add alcohol acidified slightly with hydrochloric acid and warm upon a water-bath until the solution becomes colored an emerald green.

In examining urine for bile pigments, according to Steensma, this procedure may give negative results even in the presence of the pigments, owing to the fact that the acid-alcohol is not a sufficiently strong oxidizing agent. He therefore suggests the addition of a drop of a 0.5 per cent solution of sodium nitrite to the acid-alcohol mixture before warming on the water-bath. Try this modification also.

(*e*) *Hammarsten's Reaction.*—To about 5 c.c. of Hammarsten's reagent[2] in a small evaporating dish add a few drops of diluted bile. A green color is produced. If more of the reagent is now added the play of colors as observed in Gmelin's test may be obtained.

(*f*) *Smith's Test.*—To 2–3 c.c. of diluted bile in a test-tube add carefully about 5 c.c. of dilute tincture of iodine (1:10) so that the fluids do not mix. A play of colors, *green*, *blue* and *violet*, is observed. In making this test upon the urine ordinarily only the *green* color is observed.

(*g*) *Salkowski-Schippers Reaction.*—To 10 c.c. of diluted bile in a test-tube add 5 drops of a 20 per cent solution of sodium carbonate and 10 drops of a 20 per cent solution of calcium chloride. Filter off the resultant precipitate upon a hardened filter-paper and wash it with water. Remove the precipitate to a small porcelain dish, add 3 c.c. of an acid-alcohol mixture[3] and a few drops of a dilute solution of sodium nitrite and heat. The production of a green color indicates the presence of bile pigments.

(*h*) *Bonanno's Reaction.*[4]—Place 5–10 c.c. of diluted bile in a small porcelain evaporating dish and add a few drops of Bonanno's reagent.[5] An emerald-green color will develop.

[1] Prepared by combining 99 c.c. of alcohol and 1 c.c. of *fuming* hydrochloric acid containing 4 grams of ferric chloride per liter.

[2] Hammarsten's reagent is made by mixing 1 volume of 25 per cent nitric acid and 19 volumes of 25 per cent hydrochloric acid and then adding 1 volume of this acid mixture to 4 volumes of 95 per cent alcohol.

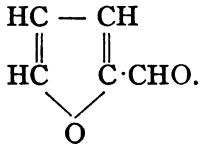
[3] Made by adding 5 c.c. of concentrated hydrochloric acid to 95 c.c. of 96 per cent alcohol.

[4] *Il Tommasi*, 2, No. 21.

[5] This reagent may be prepared by dissolving 2 grams of sodium nitrite in 100 c.c. of concentrated hydrochloric acid.

5. **Tests for Bile Acids.** (*a*) *Pettenkofer's Test.*—To 5 c.c. of diluted bile in a test-tube add 5 drops of a 5 per cent solution of sucrose. Now run about 2–3 c.c. of concentrated sulphuric acid *carefully* down the side of the tube and note the *red* ring at the point of contact. Upon slightly agitating the contents of the tube the whole solution gradually assumes a *reddish* color. As the tube becomes warm, it should be cooled in running water in order that the temperature of the solution may not rise above 70° C.

(*b*) *Mylius's Modification of Pettenkofer's Test.*—To approximately 5 c.c. of diluted bile in a test-tube add 3 drops of a very dilute (1:1000) aqueous solution of furfurol,



Now run about 2–3 c.c. of concentrated sulphuric acid carefully down the side of the tube and note the *red* ring as above. In this case, also, upon shaking the tube the whole solution is colored red. Keep the temperature of the solution below 70° C. as before.

(*c*) *Neukomm's Modification of Pettenkofer's Test.*—To a few drops of diluted bile in an evaporating dish add a trace of a dilute sucrose solution and one or more drops of dilute sulphuric acid. Evaporate on a water-bath and note the development of a *violet* color at the edge of the evaporating mixture. Discontinue the evaporation as soon as the color is observed.

(*d*) *v. Udránsky's Test.*—To 5 c.c. of diluted bile in a test-tube add 3–4 drops of a very dilute (1:1000) aqueous solution of furfurol. Place the thumb over the top of the tube and shake the tube until a thick foam is formed. By means of a small pipette add 2–3 drops of concentrated sulphuric acid to the foam and note the *dark pink* coloration produced.

(*e*) *Guerin's Reaction.*—To equal volumes of diluted bile and alcohol in a test-tube add 5–6 drops of a *saturated* aqueous solution of furfurol and 5–6 drops of concentrated sulphuric acid. A *blue* color indicates bile acids.

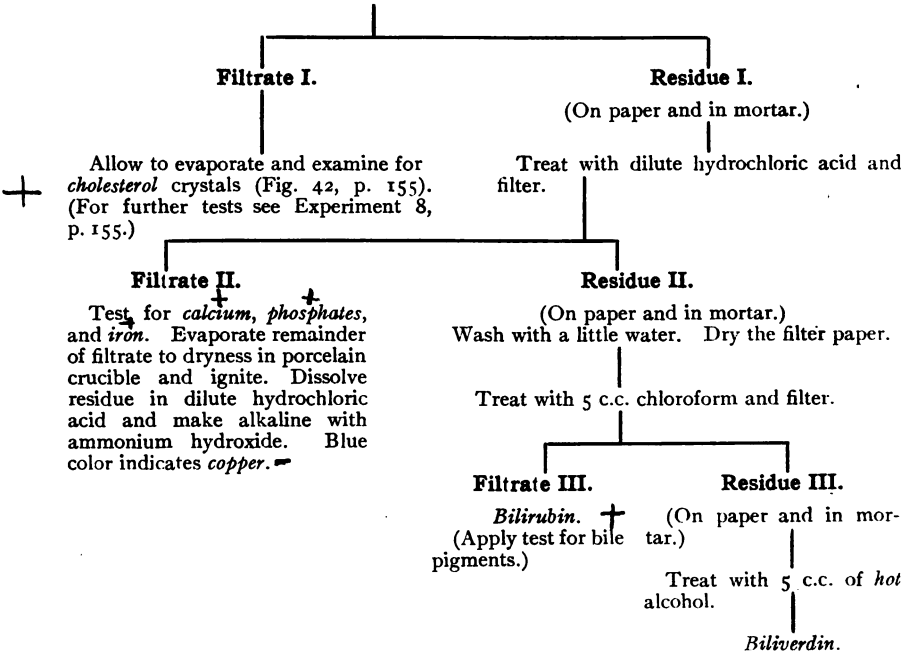
(*f*) *Hay's Test.*—This test is based upon the principle that bile acids have the property of reducing the surface tension of fluids in which they are contained. The test is performed as follows: Cool about 10 c.c. of diluted bile in a test-tube to 17° C. or lower and sprinkle

a little finely pulverized sulphur upon the surface of the fluid. The presence of bile acids is indicated if the sulphur sinks to the bottom of the liquid, the rapidity with which the sulphur sinks depending upon the quantity of bile acids present in the mixture. The test is said to react with bile acids when they are present in the proportion 1 : 120,000.

Some investigators claim that it is impossible to differentiate between bile acids and bile pigments by this test.

6. **Crystallization of Bile Salts.**—To 25 c.c. of *undiluted* bile in an evaporating dish add enough animal charcoal to form a paste and evaporate to dryness on a water-bath. Remove the residue, grind it in a mortar, and transfer it to a small flask. Add about 50 c.c. of 95 per cent alcohol and boil on a water-bath for 20 minutes. Filter, and add ether to the filtrate until there is a slight *permanent* cloudiness. Cover the vessel and stand it away until crystallization is complete. Examine the crystals under the microscope and compare them with those shown in Fig. 40, page 149. Try one of the tests for bile acids upon some of the crystals.

7. **Analysis of Biliary Calculi.**—Grind the calculus in a mortar with 10 c.c. of ether. Filter.



8. **Tests for Cholesterol.**

(*a*) *Microscopical Examination.*—Examine the crystals under the microscope and compare them with those shown in Fig. 42, below.

(*b*) *Iodine-sulphuric Acid Test.*—Place a few crystals of cholesterol in one of the depressions of a test-tablet and treat with a drop of concentrated sulphuric acid and a drop of a very dilute solution of iodine. A play of colors consisting of *violet*, *blue*, *green*, and *red* results.

(*c*) *The Liebermann-Burchard Test.*—Dissolve a few crystals of cholesterol in 2 c.c. of chloroform in a dry test-tube. Now add 10 drops of acetic anhydride and 1–3 drops of concentrated sulphuric acid. The solution becomes *red*, then *blue*, and finally *bluish-green* in color.

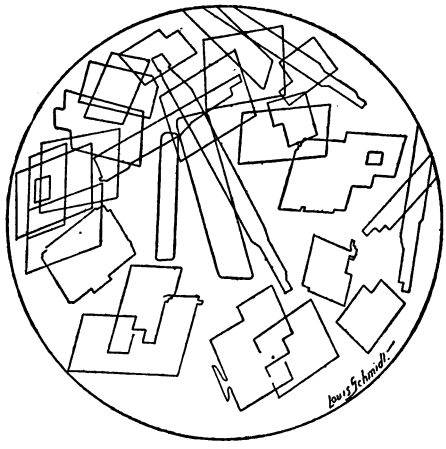


FIG. 42.—CHOLESTEROL.

(*d*) *Salkowski's Test.*—Dissolve a few crystals of cholesterol in a little choloroform and add an equal volume of concentrated sulphuric acid. A play of colors from *bluish-red* to *cherry-red* and *purple* is noted in the choloroform while the acid assumes a marked green fluorescence.

(*e*) *Schiff's Reaction.*—To a little cholesterol in an evaporating dish add a few drops of Schiff's reagent.[1] Evaporate to dryness over a low flame and observe the *reddish-violet* residue which changes to a *bluish-violet*.

9. **Preparation of Taurine.**—To 300 c.c. of bile in a casserole add 100 c.c. of hydrochloric acid and heat until a sticky mass (dyslysin) is formed. This point may be determined by drawing out a thread-like portion of the mass by means of a glass rod, and if it solidifies

[1] Schiff's reagent consists of a mixture of three volumes of concentrated sulphuric acid and one volume of 10 per cent ferric chloride.

immediately and assumes a brittle character we may conclude that all the taurocholic and glycocholic acid has been decomposed. Decant the solution and concentrate it to a small volume on the water-bath. Filter the hot solution to remove sodium chloride and other substances which may have separated, and evaporate the filtrate to dryness. Dissolve the residue in 5 per cent hydrochloric acid and precipitate with ten volumes of 95 per cent alcohol. Filter off the taurine and recrystallize it from hot water. (Save the alcoholic filtrate for the preparation of glycocoll, below.) Make the following tests upon the taurine crystals.

(*a*) Examine them under the microscope and compare with Fig. 43.

FIG. 43.—TAURINE.

(*b*) Heat a crystal upon platinum foil. The taurine at first melts, then turns brown, and finally carbonizes as the temperature is raised. Note the suffocating odor. What is it?

(*c*) Test the solubility of the crystals in water and in alcohol.

(*d*) Grind up a crystal with four times its volume of dry sodium carbonate and fuse on platinum foil. Cool the residue, transfer it to a test-tube, and dissolve it in water. Add a little dilute sulphuric acid and note the odor of hydrogen sulphide. Hold a piece of filter paper, moistened with a *small* amount of lead acetate, over the opening of the test-tube and observe the formation of lead sulphide.

10. **Preparation of Glycocoll.**—Concentrate the alcoholic filtrate from the last experiment (9) until no more alcohol remains. The glycocoll is present here in the form of an hydrochloride and may be liberated from this combination by the addition of freshly precipi-

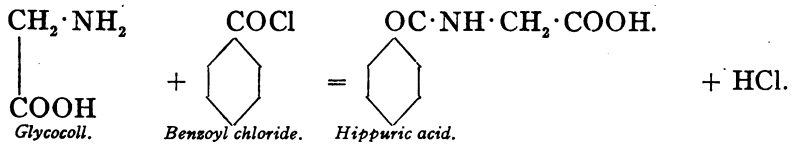
tated lead hydroxide or by lead hydroxide solution. Remove the lead by hydrogen sulphide. Filter and decolorize the filtrate by animal charcoal. Filter again, concentrate the filtrate, and set it aside for crystallization. Glycocoll separates as colorless crystals (Fig. 44).

11. **Synthesis of Hippuric Acid.**—To some of the glycocoll prepared in the last experiment or furnished by the instructor, add a little water, about 1 c.c. of benzoyl chloride and render alkaline with potassium hydroxide solution. Stopper the tube and shake it until no more heat is evolved. Now render strongly alkaline with potassium hydroxide and shake the mixture until no odor of benzoyl chloride can be detected. Cool, acidify with hydrochloric acid, add an equal

FIG. 44.—GLYCOCOLL.

volume of petroleum ether, and shake thoroughly to remove the benzoic acid. (Evaporate this solution and note the crystals of benzoic acid. Compare them with those shown in Fig. 94, page 284.) Decant the ethereal solution into a porcelain dish and extract again with ether. The hippuric acid remains in the aqueous solution. Filter it off and wash it with a small amount of cold water while still on the filter. Remove it to a small, shallow vessel, dissolve it in a small amount of hot water and set it aside for crystallization. Examine the crystals microscopically and compare them with those in Fig. 92, page 276.

The chemistry of the synthesis is represented thus:

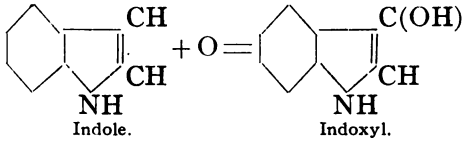


CHAPTER X.

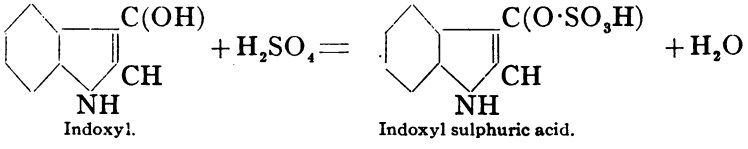
PUTREFACTION PRODUCTS.

THE putrefactive processes in the intestine are the result of the action of bacteria upon the protein material present. This bacterial action which is the combined effort of many forms of micro-organisms is confined almost exclusively to the large intestine. Some of the products of the putrefaction of proteins are identical with those formed in tryptic digestion, although the decomposition of the protein material is much more extensive when subjected to putrefaction. Some of the more important of the putrefaction products are the following: *Indole, skatole, paracresol, phenol, para-oxyphenylpropionic acid, para-oxyphenylacetic acid, volatile fatty acids, hydrogen sulphide, methane, methyl mercaptan, hydrogen,* and *carbon dioxide,* beside *proteoses, peptones, ammonia,* and *amino acids.* Of these the indole, skatole, phenol, and paracresol appear in part in the urine as ethereal sulphuric acids, whereas the oxyacids mentioned pass unchanged into the urine. The potassium indoxyl sulphate (page 273) content of the urine is a rough indicator of the extent of the putrefaction within the intestine.

The portion of the indole which is excreted in the urine is first subjected to a series of changes within the organism and is subsequently eliminated as *indican.* These changes may be represented thus:

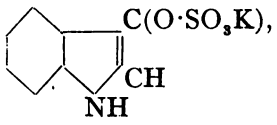


Indole. Indoxyl.



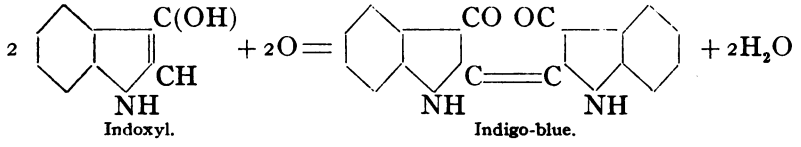
Indoxyl. Indoxyl sulphuric acid.

In the presence of potassium salts the indoxyl sulphuric acid is then transformed into indoxyl potassium sulphate (or indican),



and eliminated as such in the urine.

Indican may be decomposed by treatment with concentrated hydrochloric acid (see tests on page 275) into sulphuric acid and indoxyl. The latter body may then be oxidized to form indigo-blue thus:



Indoxyl. Indigo-blue.

This same reaction may also occur under pathological conditions *within the organism*, thus giving rise to the appearance of crystals of indigo-blue in the urine.

Skatole is likewise changed within the organism and eliminated in the form of a chromogenic substance. Skatole is, however, of less importance as a putrefaction product than indole and ordinarily occurs in much smaller amount. The tryptophane group of the protein molecule yields the indole and skatole formed in intestinal putrefaction, but the reasons for the transformation of the major portion of this tryptophane into indole and the minor portion into skatole are not well understood. Indole is more toxic than skatole.

Phenol occurs in fairly large amount in certain abnormal conditions of the organism, but ordinarily the amount is very small. It is probably derived from the tyrosine group of the protein molecule. Phenol is conjugated in the liver to form phenyl potassium sulphate and appears in the urine in this form (Baumann and Herter). Para-cresol occurs in the urine as cresyl potassium sulphate.

Regarding the claim of Nencki that methyl mercaptan is formed as a gas during intestinal putrefaction it is an important fact that Herter[1] has been unable to detect the mercaptan in *fresh* feces. He is, therefore, not inclined to accept the theory that methyl mercaptan is formed in *ordinary* intestinal putrefaction but believes that it may be formed in *exceptional cases*. Hydrogen sulphide is, however, formed in all cases of intestinal putrefaction.

[1] Herter: *Bacterial Infections of the Digestive Tract*, p. 227.

Experiments on Putrefaction Products.

In many courses in physiological chemistry the instructors are so limited for time that no extended study of the products of putrefaction can very well be attempted. Under such conditions the scheme here submitted may be used profitably in the way of a demonstration. Where the number of students is not too great, a single large putrefaction may be started, and, after the initial distillation, both the resulting distillate and residue may be distributed to the members of the class for individual manipulation.

Preparation of Putrefaction Mixture.—Place a weighed mixture of coagulated egg albumin and ground lean meat in a flask or bottle and add approximately 2 liters of water for every kilogram of protein used. Sterilize the vessel and contents, inoculate with the *colon bacillus*, and keep at 40° C. for two or three weeks. If cultures of the colon bacillus are not available, add 60 c.c. of a cold saturated solution of sodium carbonate for every liter of water previously added and inoculate with some putrescent material (pancreas or feces).[1] Mix the putrefaction mixture very thoroughly by shaking and insert a cork furnished with a glass tube to which is attached a wash bottle containing a 3 per cent solution of mercuric cyanide.[2] This device is for the purpose of collecting the methyl mercaptan, a gas formed during the process of putrefaction. It also serves to diminish the odor arising from the putrefying material. Place the putrefaction mixture at 40° C. for two or three weeks and at the end of that time make a separation of the products of putrefaction according to the following directions:

Subject the mixture to distillation until the distillate and residue are approximately equal in volume.

[1] Putrefying protein may be prepared by treating 10 grams of finely ground lean meat with 100 c.c. of water and 2 c.c. of a saturated solution of sodium carbonate and keeping the mixture at 40° C. for twenty-four hours.

[2] Concentrated sulphuric acid containing a small amount of *isatin* may be used as a substitute for mercuric cyanide. When this modification is employed it is necessary to use calcium chloride tubes to exclude moisture from the isatin solution.

PART I.

MANIPULATION OF THE DISTILLATE.

Acidify with hydrochloric acid and extract with ether.

Ether Extract No. 1.
Add an equal volume of water, make alkaline with potassium hydroxide, and shake thoroughly.

Residue No. 1
Allow the ether to volatilize. Evaporate and detect *ammonium chloride* crystals (Fig. 45, p. 162).

Ether Extract No. 2.
Evaporate spontaneously. *Indole* and *skatole* remain. Try proper reactions (see pages 164 and 166).

Alkaline Solution No. 1.
Acidify with hydrochloric acid, add sodium carbonate, and extract with ether.

Ether Extract No. 3.
Evaporate. Detect *phenol* and *cresol* (paracresol). See p. 166.

Alkaline Solution No. 2.
Acidify with hydrochloric acid, and extract with ether.

Ether Extract No. 4.
Evaporate. *Volatile fatty acids* remain.

Final Residue.
(Discard.)

DETAILED DIRECTIONS FOR MAKING THE SEPARATIONS INDICATED IN THE SCHEME.

Preliminary Ether Extraction.—This extraction may be conveniently conducted in a separatory funnel. Mix the fluids for extraction in the ratio of *two* volumes of ether to *three* volumes of the distillate. Shake very thoroughly for a few moments, then draw off the extracted fluid and add a new portion of the distillate. Repeat the process until the entire distillate has been extracted. Add a small amount of fresh ether at each extraction to replace that dissolved by the water in the preceding extraction.

Residue No. 1.—Unite the portions of the distillate extracted as above and allow the ether to volatilize spontaneously. Evaporate until crystallization begins. Examine the crystals under the microscope. Ammonium chloride predominates. Explain its presence.

Ether Extract No. 1.—Add an equal volume of water, render the mixture alkaline with potassium hydroxide, and shake thoroughly by means of a separatory funnel as before. The *volatile fatty acids*, contained among the putrefaction products, would be dissolved by the alkaline solution (No. 1) whereas any indole or skatole would remain in the ethereal solution (No. 2).

FIG. 45.—AMMONIUM CHLORIDE.

Alkaline Solution No. 1.—Acidify with hydrochloric acid and add sodium carbonate solution until the fluid is neutral or slightly acid from the presence of carbonic acid. At this point a portion of the solution, after being heated for a few moments, should possess an alkaline reaction on cooling. Extract the whole mixture with ether in the usual way, using care in the manipulation of the stop cock to relieve the pressure due to the evolution of carbon dioxide. The ether (Ether Extract No. 3) removes any *phenol* or *cresol* which may be present while the volatile fatty acids will remain in the alkaline solution (No. 2) as alkali salts.

Ether Extract No. 2.—Drive off the major portion of the ether at a low temperature on a water-bath and allow the residue to evaporate spontaneously. Indole and skatole should be present here. Prove the presence of these bodies. For tests for indole and skatole see pp. 164 and 166.

Alkaline Solution No. 2.—Make strongly acid with hydrochloric acid and extract with a small amount of ether, using a separatory funnel. As carbon dioxide is liberated here, care must be used in the manipulation of the stop cock of the funnel in relieving the pressure

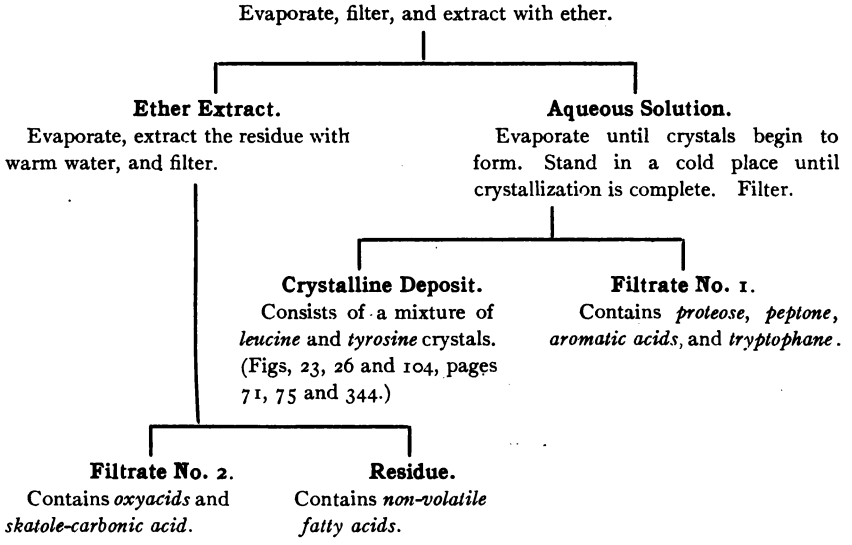
within the vessel. The volatile fatty acids are dissolved by the ether (Ether Extract No. 4).

Ether Extract No. 3.—Evaporate this ethereal solution on a water-bath. The oily residue contains phenol and cresol. The cresol is present for the most part as paracresol. Add some water to the oily residue and heat it in a flask. Cool and prove the presence of phenol and cresol. For tests for these bodies see page 166.

Ether Extract No. 4.—Evaporate on a water-bath. The volatile fatty acids remain in the residue.

PART II.

MANIPULATION OF THE RESIDUE.



DETAILED DIRECTIONS FOR MAKING THE SEPARATIONS INDICATED IN THE SCHEME.

Preliminary Ether Extraction.—This extraction may be conducted in a separatory funnel. In order to make a satisfactory extraction the mixture should be shaken very thoroughly. Separate the ethereal solution from the aqueous portion and treat them according to the directions given on p. 161.

Ether Extract.—Evaporate this solution on a *safety* water-bath until the ether has been entirely removed. Extract the residue with warm water and filter.

Aqueous Solution.—Evaporate this solution until crystallization begins. Stand the solution in a cold place until no more crystals form. This crystalline mass consists of impure leucine and tyrosine. Filter off the crystals.

Crystalline Deposit.—Examine the crystals under the microscope and compare them with those reproduced in Figs. 23, 26, and 104, pages 71, 75 and 344. Do the forms of the crystals of leucine and tyrosine resemble those previously examined? Make a separation of the leucine and tyrosine and apply typical tests according to directions given on pages 81 and 82.

Filtrate No. 1.—Make a test for tryptophane with bromine water (see page 142), and also with the Hopkins-Cole reagent (see page 89). Use the remainder of the filtrate for the separation of proteoses and peptones. Make the separation according to the directions given on page 112.

Filtrate No. 2.—This solution contains para-oxyphenylacetic acid, para-oxyphenylpropionic acid and skatole-carbonic acid. Prove the presence of these bodies by appropriate tests. Tests for oxyacids and skatole-carbonic acid are given on page 167.

TESTS FOR VARIOUS PUTREFACTION PRODUCTS.

Tests for Indole.

1. **Herter's β-Naphthaquinone Reaction.**—(*a*) To a dilute aqueous solution of indole (1:500,000) add one drop of a 2 per cent solution of β-naphthaquinone-sodium-monosulphonate. No reaction occurs. Add a drop of a 10 per cent solution of potassium hydroxide and note the gradual development of a blue or blue-green color which fades to green if an excess of the alkali is added. Render the green or blue-green solution acid and note the appearance of a pink color. Heat facilitates the development of the color reaction.

One part of indole in *one million parts* of water may be detected by means of this test if carefully performed.

(*b*) If the alkali be added to a more concentrated indole solution *before* the introduction of the naphthaquinone the course of the reaction is different, particularly if the indole solution is somewhat more concentrated than that mentioned above and if heat is used.

Under these conditions the blue indole compound ultimately forms as fine acicular crystals which rise to the surface.

If we do not wait for the production of the crystalline body but as soon as the blue color forms, shake the aqueous solution with chloroform, the blue color disappears from the solution and the chloroform assumes a *pinkish-red hue.* This is a distinguishing feature of the indole reaction and facilitates the differentiation of indole from other bodies which yield a similar blue color.

2. **Konto's Reaction.**—Distil the solution to be tested until only one-third of the original solution remains. Make the distillate alkaline with sodium hydroxide and distil again in order to separate the indole from the phenol, the latter remaining in the residue. Inasmuch as this second distillate generally contains a large amount of ammonia it should be acidified with dilute sulphuric acid and again distilled. To 1 c.c. of this ammonia-free distillate in a test-tube add 3 drops of a 40 per cent solution of formaldehyde and 1 c.c. of concentrated sulphuric acid. Now agitate the mixture and note the appearance of a violet red color if a trace of indole is present. The test is said to serve for the detection of indole when present in a dilution of 1:700,000.

Skatole gives a yellow or brown color under the above conditions.

3. **Cholera-red Reaction.**—To a little of the residue in a test-tube add one-tenth its volume of a 0.02 per cent solution of potassium nitrite and mix thoroughly. Carefully run concentrated sulphuric acid down the side of the tube so that it forms a layer at the bottom. Note the purple color. Neutralize with potassium hydroxide and observe the production of a bluish-green color.

4. **Legal's Reaction.**—To a small amount of the residue in a test-tube add a few drops of a freshly prepared solution of sodium nitroprusside, $Na_2Fe(CN)_5NO + 2H_2O$. Render alkaline with potassium hydroxide and note the production of a violet color. If the solution is now acidified with glacial acetic acid the violet is transformed into a blue.

5. **Pine Wood Test.**—Moisten a pine splinter with concentrated hydrochloric acid and insert it into the residue. The wood assumes a cherry-red color.

6. **Nitroso-indole Nitrate Test.**—Acidify some of the residue with nitric acid, add a few drops of a potassium nitrite solution and note the production of a red precipitate of nitroso-indole nitrate. If the residue contains but little indole simply a red coloration will result. Compare this result with the result of the similar test on skatole.

Tests for Skatole.

1. **Herter's Para-dimethylaminobenzaldehyde Reaction.**[1]—To 5 c.c. of the distillate or aqueous solution under examination add 1 c.c. of an acid solution of para-dimethylaminobenzaldehyde[2] and heat the mixture to boiling. A purplish-blue coloration is produced[3] which may be intensified through the addition of a few drops of concentrated hydrochloric acid. If the solution be cooled under running water it loses its purplish tinge of color and becomes a definite blue. The solution at this point may be somewhat opalescent through the separation of uncombined para-dimethylaminobenzaldehyde. Care should be taken not to add an excess of hydrochloric acid inasmuch as the end-reaction has a tendency to fade under the influence of a high acidity.

A rough idea regarding the actual quantity of skatole in a mixture may be obtained by extracting this blue solution with chloroform and subsequently comparing this chloroform solution, by means of a colorimeter (Duboscq), with the maximal reaction, obtained with a skatole solution of known strength.

2. **Color Reaction with Hydrochloric Acid.**—Acidify some of the residue with concentrated hydrochloric acid. Note the production of a violet color.

3. Acidify some of the residue with nitric acid and add a few drops of a potassium nitrite solution. Note the white turbidity. Compare this result with the result of the similar test on indole.

Tests for Phenol and Cresol.

1. **Color Test.**—Test a little of the solution with Millon's reagent. A red color results. Compare this test with the similar one under Tyrosine (see page 81).

2. **Ferric Chloride Test.**—Add a few drops of *neutral* ferric chloride solution to a little of the residual fluid. A dirty bluish-gray color is formed.

3. **Formation of Bromine Compounds.**—Add some bromine water to a little of the fluid under examination. Note the crystalline precipitate of tribromphenol and tribromcresol.

[1] Herter: *Bacterial Infections of the Digestive Tract*, 1907, p. 141.

[2] Made by dissolving 5 grams of para-dimethylaminobenzaldehyde in 100 c.c. of 10 per cent sulphuric acid.

[3] If the color does not appear add more of the aldehyde solution.

Tests for Oxyacids.

1. **Color Test.**—Test a little of the solution with Millon's reagent. A red color results.

2. **Bromine Water Test.**—Add a few drops of bromine water to some of the filtrate. A turbidity or precipitate is observed.

Test for Skatole-carbonic Acid.

Ferric Chloride Test.—Acidify some of the filtrate with hydrochloric acid, add a few drops of ferric chloride solution, and heat. Compare the end-reaction with that given by phenol.

CHAPTER XI.

FECES.

THE feces is the residual mass of material remaining in the intestine after the full and complete exercise of the digestive and absorptive functions and is ultimately expelled from the body through the rectum. The amount of this fecal discharge varies with the individual and the diet. Upon an ordinary mixed diet the daily excretion by an adult male will aggregate 110–170 grams with a solid content ranging between 25 and 45 grams; the fecal discharge of such an individual

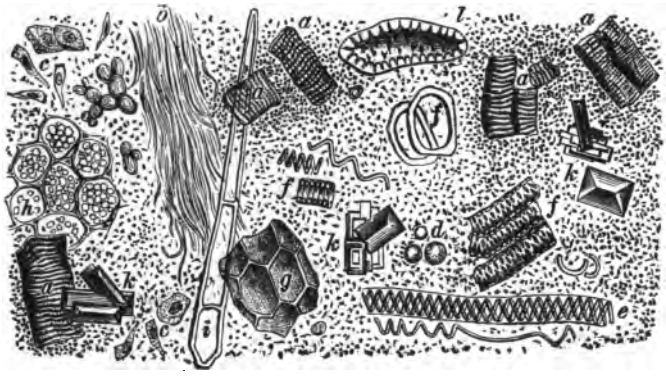


FIG. 46.—MICROSCOPICAL CONSTITUENTS OF FECES. (*v. Jaksch.*)

a, Muscle fibers; *b*, connective tissue; *c*, epithelium; *d*, leucocytes; *e*, spiral cells; *f*, *g*, *h*, *i*, various vegetable cells; *k*, "triple phosphate" crystals; *l*, woody vegetable cells; the whole interspersed with innumerable micro-organisms of various kinds.

upon a vegetable diet will be much greater and may even be as great as 350 grams and possess a solid content of 75 grams. The variation in the normal daily output being so great renders this factor of very little value for diagnostic purposes, except where the composition of the diet is accurately known. Lesions of the digestive tract, a defective absorptive function, or increased peristalsis as well as an admixture of mucus, pus, blood, and pathological products of the intestinal wall may cause the total amount of excrement to be markedly increased.

The fecal pigment of the normal adult is hydrobilirubin. This pigment originates from the bilirubin which is secreted into the intes-

tine in the bile, the transformation from bilirubin to hydrobilirubin being brought about through the activity of certain bacteria. Hydrobilirubin is sometimes called stercobilin and bears a close resemblance to urobilin or may even be identical with that pigment. Neither bilirubin nor biliverdin occurs normally in the fecal discharge of adults, although the former may be detected in the excrement of nursing infants. The most important factor, however, in determining the color of the fecal discharge is the diet. A mixed diet, for instance, produces stools which vary in color from light to dark brown, an exclusive meat diet gives rise to a brownish-black stool, whereas the stool resulting from a milk diet is invariably light colored. Certain pigmented foods such as the chlorophyllic vegetables, and various varieties of berries, each afford stools having a characteristic color. Certain drugs act in a similar way to color the fecal discharge. This is well illustrated by the occurrence of green stools following the use of calomel and of black stools after bismuth ingestion. The green color of the calomel stool is generally believed to be due to biliverdin. v. Jaksch, however, claims to have proven this view to be incorrect since he was able to detect hydrobilirubin (or urobilin) but *no biliverdin* in stools after the administration of calomel. The bismuth stool derives its color from the black sulphide which is formed from the subnitrate of bismuth. In cases of biliary obstruction the grayish-white *acholic* stool is formed.

FIG. 47.—HÆMATOIDIN CRYSTALS FROM ACHOLIC STOOLS. (*v. Jaksch.*) Color of crystals same as the color of those in Fig. 41, p. 150.

Under normal conditions the odor of feces is due to skatole and indole, two bodies formed in the course of putrefactive processes occurring within the intestine (see page 158). Such bodies as methane, methyl mercaptan, and hydrogen sulphide may also add to the disagreeable character of the odor. The intensity of the odor depends to a large degree upon the character of the diet, being very marked in stools from a meat diet, much less marked in stools from a vegetable diet, and frequently hardly detectable in stools from a milk diet. Thus the stool of the infant is ordinarily nearly odorless and any decided odor may generally be readily traced to some pathological source.

A neutral reaction ordinarily predominates in normal stools

although slightly alkaline or even acid stools are met with. The acid reaction is encountered much less frequently than the alkaline and then commonly only following a vegetable diet.

The form and consistency of the stool is dependent, in large measure, upon the nature of the diet and particularly upon the quantity of water ingested. Under normal conditions the consistency may vary from a thin, pasty discharge to a firmly formed stool. Stools which are exceedingly thin and watery ordinarily have a pathological significance. In general the feces of the carnivorous animals is of a firmer consistency than that of the herbivora.

FIG. 48.—CHARCOT-LEYDEN CRYSTALS.

It is frequently desirable for clinical or experimental purposes to make an examination of the fecal output which constitutes the residual mass from a certain definite diet. Under such conditions, it is customary to cause the person under observation to ingest some substance, at the beginning and end of the period in question, which shall sufficiently differ in color and consistency from the surrounding feces as to render comparatively easy the differentiation of the feces of that period from the feces of the immediately preceding and succeeding periods. One of the most satisfactory methods of making this "separation" is by means of the ingestion of a gelatin capsule containing about 0.2 gram of powdered charcoal at the beginning and end of the period under observation. This procedure causes the appearance of *two black zones* of charcoal in the fecal mass and thus renders comparatively simple the differentiation of the feces of the intermediate period. Some similar method for the "separation of feces" is universally practised in connection with the scientifically accurate type of nutrition or metabolism experiment which embraces the collection of useful data regarding the income and outgo of nitrogen, and other elements.

Among the macroscopical constituents of the feces may be mentioned the following: Intestinal parasites, undigested food particles, gall stones, pathological products of the intestinal wall, enteroliths, intestinal sand, and objects which have been accidentally swallowed.

The fecal constituents which at various times and under different conditions may be detected by the use of the microscope are as follows: Constituents derived from the food, such as *muscle fibers*, *connective-tissue shreds*, *starch granules*, and *fat;* formed elements derived from the intestinal tract, such as *epithelium*, *erythrocytes*, and *leucocytes; mucus; pus corpuscles; parasites* and *bacteria.* In addition to the con-

stituents named the following *crystalline deposits* may be detected: *cholesterol, soaps, fatty acid, fat, bismuth sulphide, hæmatoidin, "triple phosphate," Charcot-Leyden crystals,* and the *oxalate, carbonate, phosphate, sulphate,* and *lactate* of calcium.

The detection of minute quantities of blood in the feces ("occult blood") has recently become a recognized aid to a correct diagnosis of certain disorders. In these instances the hemorrhage is ordinarily so slight that the identification by means of macroscopical characteristics as well as the microscopical identification through the detection of erythrocytes are both unsatisfactory in their results. Of the tests given for the detection of "occult blood" the *aloin-turpentine test* (page 173) is probably the most satisfactory. Since "occult blood" occurs with considerable regularity and frequency in gastrointestinal cancer and in gastric and duodenal ulcer, its detection in the feces is of especial value as an aid to a correct diagnosis of these disorders.

It has been quite clearly shown that the intestine of the newly born is sterile. However, this condition is quickly altered and bacteria may be present in the feces before or after the first ingestion of food. There are three possible means of infecting the intestine, *i. e.*, by way of the mouth or anus or through the blood. The infection by means of the blood seldom occurs except under pathological conditions, thus limiting the general infection to the mouth and anus.

In infants with pronounced constipation two-thirds of the dry substance of the stools has been found to consist of bacteria. In the stools of normal adults probably about one-third of the dry substance is bacteria.[1] The average excretion of dry bacteria in twenty-four hours for an adult is about 8 grams.

Some of the more important organisms met with in the feces are the following:[2] *B. coli, B. lactis aërogenes, Bact. Welchii, B. bifidus,* and *coccal forms.* Of these the first three types mentioned are *gas-forming* organisms. The production of gas by the fecal flora in dextrose-bouillon is subject to great variations under pathological conditions: alterations in the diet of normal persons will also cause wide fluctuations. In this connection Herter has observed a marked reduction or even complete cessation of gas production by the mixed fecal bacteria while considerable doses of benzoate were being given. A return to the former plane of gas production followed the discontinuation of the benzoate.[3] Data as to the production of gas are of

[1] Schittenhelm and Tollens found bacteria to comprise 42 per cent of the dry matter. This value is, however, probably too high.

[2] Herter and Kendall: *Journal of Biological Chemistry,* 1908, V, p. 283.

[3] Private communication from Professor C. A. Herter.

considerable importance in a diagnostic way although the exact cause of the variations is not yet established. It should be borne in mind in this connection that gas volumes are frequently variable with the same individual. For this reason it is necessary in every instance to follow the gas production for a considerable period of time before drawing conclusions.[1]

For diagnostic purposes the macroscopical and microscopical examinations of the feces ordinarily yield much more satisfactory data than are secured from its chemical examination.

Experiments on Feces.

1. **Macroscopical Examination.**—If the stool is watery pour it into a shallow dish and examine directly. If it is firm or pasty it should be treated with water and carefully stirred before the examination for macroscopical constituents is attempted.

The macroscopical constituents may be collected very satisfactorily by means of a Boas sieve (Fig. 49). This sieve is constructed of two easily detachable hemispheres which are held together by means of a bayonet catch. In using the apparatus the feces is spread out upon a very fine sieve contained in the lower hemisphere and a stream of water is allowed to play upon it through the medium of an opening in the upper hemisphere. The apparatus is provided with an orifice in the upper hemisphere through which the feces may be stirred by means of a glass rod during the washing process. After 15–30 minutes' washing nothing but the coarse fecal constituents remain upon the sieve.

Fig. 49.—Boas' Sieve.

2. **Microscopical Examination.**—Watery stools should be placed in a shallow dish, thoroughly mixed, and a small amount removed to a slide for examination. Stools of a firm or pasty consistency should be rubbed up in a mortar with physiological salt solution and a small portion of the resulting mixture transferred to a slide for examination. In normal feces look for *food particles*, *bacteria*, and *crystalline bodies*. In pathological stools, in addition to these substances, look for *animal parasites* and *pathological products* of the intestinal wall. See Fig. 46, page 168.

[1] Herter and Kendall: *loc. cit.*

2. **Reaction.**—Thoroughly mix the feces and apply moist red and blue litmus papers to the surface. If the stool is hard it should be mixed with water before the reaction is taken. Examine the stool as soon after defecation as is convenient, since the *reaction* may change very rapidly. The *reaction* of the normal stools of adult man is ordinarily neutral or faintly alkaline to litmus, but seldom acid. Infants' stools are generally acid in reaction.

4. **Starch.**—If any imperfectly cooked starch-containing food has been ingested it will be possible to detect starch granules by a microscopical examination of the feces. If the granules are not detected by a microscopical examination, the feces should be placed in an evaporating dish or casserole and boiled with water for a few minutes. Filter and test the filtrate by the iodine test in the usual way (see page 45).

5. **Cholesterol and Fat.**—Extract the *dry* feces with ether in a Soxhlet apparatus (see Fig. 126). If this apparatus is not available transfer the dry feces to a flask, add ether, and shake frequently for a few hours. Filter and remove the ether by evaporation. The residue contains cholesterol and the mixed fats of the feces. For every gram of fat add about 1 1/2 gram of solid potassium hydroxide and 25 c.c. of 95 per cent alcohol and boil in a flask on a water-bath for one-half hour, maintaining the volume of alcohol constant. This alcoholic-potash has saponified the mixed fats and we now have a mixture of soaps and cholesterol. Add sodium chloride, in substance, to the mixture and extract with ether to dissolve out the cholesterol. Remove the ether by evaporation and examine the residue microscopically for cholesterol crystals. Try any of the other tests for cholesterol as given on page 155.

6. **Blood.**—Undecomposed blood may be detected macroscopically. If uncertain, look for erythrocytes under the microscope, and spectroscopically for the spectrum of oxyhæmoglobin (see Absorption Spectra, Plate I).

In case the blood has been altered or is present in minute amount ("occult blood"), and cannot be detected by the means just mentioned, the following tests may be tried:

(*a*) *Aloin-turpentine Test.*—Mix the stool very thoroughly and take about 5 grams of the mixture for the test. Reduce this sample to a semi-fluid mass by means of distilled water and extract very thoroughly with an equal volume of ether to remove any fat which may be present. Now treat the extracted feces with one-third its volume of glacial acetic acid and 10 c.c. of ether and extract very

thoroughly as before. The acid-ether extract will rise to the top and may be removed.

Introduce 2–3 c.c. of this acid-ether solution into a test-tube, add an equal volume of a dilute solution of aloin in 70 per cent alcohol and 2–3 c.c. of ozonized turpentine and shake the tube gently. If blood is present the entire volume of fluid ordinarily becomes pink and finally cherry-red. In some instances the color will be limited to the aloin solution which sinks to the bottom. This color reaction should occur within fifteen minutes in order to indicate a positive test for blood, since the aloin will turn red of itself if allowed to stand for a longer period. The color is ordinarily light yellow in a negative test. Hydrogen peroxide is not a satisfactory substitute for turpentine in the test.

(*b*) *Weber's Guaiac Test.*—Mix a little feces with 30 per cent acetic acid to form a fluid mass. Transfer to a test-tube and extract with ether. If blood is present the ether will assume a brownish-red color. Filter off the ether extract and to a portion of the filtrate add an alcoholic solution of guaiac (strength about 1 : 60),[1] drop by drop, until the fluid becomes turbid. Now add hydrogen peroxide or old turpentine. In the presence of blood a blue color is produced (see page 191).

(*c*) *Cowie's Guaiac Test.*—To 1 gram of moist feces add 4–5 c.c. of glacial acetic acid and extract the mixture with 30 c.c. of ether. To 1–2 c.c. of the extract *add an equal volume of water*, agitate the mixture, introduce a few granules of powdered guaiac resin, and after bringing the resin into solution, gradually add 30 drops of old turpentine or hydrogen peroxide. A blue color indicates the presence of blood. Cowie claims that by means of this test an intestinal hemorrhage of 1 gram can easily be detected by an examination of the feces.

(*d*) *Acid-hæmatin.*—Examine some of the ethereal extract from Experiment (*b*) spectroscopically. Note the typical spectrum of acid-hæmatin (see Absorption Spectra, Plate II).

7. **Hydrobilirubin.** *Schmidt's Test.*—Rub up a small amount of feces in a mortar with a concentrated aqueous solution of mercuric chloride. Transfer to a shallow, flat-bottomed dish and allow to stand 6–24 hours. The presence of hydrobilirubin will be indicated by a deep red color being imparted to the particles of feces containing this pigment. This red color is due to the formation of hydrobilirubin-

[1] Buckmaster advises the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiac resin.

mercury. If unaltered bilirubin is present in any portion of the feces that portion will be green in color due to the oxidation of bilirubin to biliverdin.

Another method for the detection of hydrobilirubin is the following: Treat the dry feces with absolute alcohol acidified with sulphuric acid and shake thoroughly. The acidified alcohol extracts the pigment and assumes a reddish color. Examine a little of this fluid spectroscopically and note the typical spectrum of hydrobilirubin (Absorption Spectra, Plate II).

8. **Bilirubin.**[1] (*a*) *Gmelin's Test.*—Place a few drops of concentrated nitric acid in an evaporating dish or on a porcelain test-tablet and allow a few drops of the feces and water to mix with it. The usual play of colors of Gmelin's test is produced, *i. e.*, green, blue, violet, red, and yellow. If so desired, this test may be executed on a slide and observed under a microscope.

(*b*) *Huppert's Test.*—Treat the feces with water to form a semi-fluid mass, add an equal amount of milk of lime, shake thoroughly, and filter. Wash the precipitate with water, then transfer both the paper and the precipitate to a small beaker or flask, add a small amount of 95 per cent alcohol acidified slightly with sulphuric acid, and heat to boiling on a water-bath. The presence of bilirubin is indicated by the alcohol assuming a green color.

Steensma advises the addition of a drop of a 0.5 per cent solution of sodium nitrite to the acid-alcohol mixture before warming on the water-bath. Try this modification also.

9. **Bile Acids.**—Extract a small amount of feces with alcohol and filter. Evaporate the filtrate on a water-bath to drive off the alcohol and dissolve the residue in water made slightly alkaline with potassium hydroxide. Upon this aqueous solution try any of the tests for bile acids given on page 153.

10. **Caseinogen.**—Extract the fresh feces first with a dilute solution of sodium chloride, and later with water acidified with dilute acetic acid, to remove soluble proteins. Now extract the feces with 0.5 per cent sodium carbonate and filter. Add dilute acetic acid to the filtrate to precipitate the caseinogen, being careful not to add an excess of the reagent as the caseinogen would dissolve. Filter off the caseinogen and test it according to directions given on page 219. Caseinogen is found principally in the feces of children who have been fed a milk diet. Mucin would also be extracted by the dilute alkali, if

[1] The detection of bilirubin in the feces is comparatively simple provided it is not accompanied by other pigments. When other pigments are present, however, it is difficult to detect the bilirubin and, at times, may be found impossible.

present in the feces. What test could you make on the newly precipitated body to differentiate between mucin and caseinogen?

11. **Nucleoprotein.**—Mix the stool thoroughly with water, transfer to a flask, and add an equal amount of saturated lime water. Shake frequently for a few hours, filter, and precipitate the nucleoprotein with acetic acid. Filter off this precipitate and test it as follows:

(*a*) *Phosphorus.*—Test for phosphorus by fusion (see page 247).

(*b*) *Solubility.*—Try the solubility in the ordinary solvents.

(*c*) *Protein Color Test.*—Try any of the protein color tests.

What proof have you that the above body was not mucin? What other test can you use to differentiate between nucleoprotein and mucin?

12. **Albumin and Globulin.**—Extract the fresh feces with a dilute solution of sodium chloride. (The preliminary extract from the preparation of caseinogen (10), above, may be utilized here.) Filter, and saturate a portion of the filtrate with sodium chloride in substance. A precipitate signifies globulin. Filter off the precipitate and acidify the filtrate slightly with dilute acetic acid. A precipitate at this point signifies albumin. Make a protein color test on each of these bodies.

13. **Proteose and Peptone.**—Heat to boiling the portion of the sodium chloride extract not used in the last experiment. Filter off the coagulum, if any forms. Acidify the filtrate slightly with acetic acid and saturate with sodium chloride in substance. A precipitate here indicates proteose. Filter it off and test it according to directions given on page 111. Test the filtrate for peptone by the biuret test.

14. **Inorganic Constituents.**—Prepare a dilute aqueous solution of dry feces and decolorize it by means of purified animal charcoal. Make the following tests upon the clear solution.

(*a*) *Chlorides.*—Acidify with nitric acid and add argentic nitrate.

(*b*) *Phosphates.*—Acidify with nitric acid, add molybdic solution, and warm gently.

(*c*) *Sulphates.*—Acidify with hydrochloric acid, add barium chloride, and warm.

15. **Konto's Reaction for Indole.**—Rub up the stool with water to form a thin paste. From this point the test is the same as for the detection of indole in putrefaction mixtures (see page 165).

16. **Schmidt's Nuclei Test.**—This test serves as an aid to the diagnosis of pancreatic insufficiency. The test is founded upon the theory that cell nuclei are digestible *only* in pancreatic juice, and there-

fore that the appearance in the feces of such nuclei indicates insufficiency of pancreatic secretion. The procedure is as follows: Cubes of fresh beef about one-half centimeter square are enclosed in small gauzè bags and ingested with a test meal. Subsequently the fecal mass resulting from this test-meal is examined, the bag opened, and the condition of the enclosed residue determined. Under normal conditions the nuclei would be digested. Therefore if the nuclei are found to be for the most part undigested, and the intervening period has been sufficient to permit of the full activity of the pancreatic function (at least six hours), it may be considered a sign of pancreatic insufficiency.

It has been claimed by Steele that under certain conditions the non-digestion of the nuclei may indicate a general lowering of the digestive power rather than a true pancreatic insufficiency.

CHAPTER XII.

BLOOD.

BLOOD is composed of four types of form-elements (erythrocytes or red blood corpuscles, leucocytes or white blood corpuscles, blood plates or plaques and blood dust or hæmoconien) held in suspension in a fluid called *blood plasma*. These form-elements compose about 60 per cent of the blood, by weight. Ordinarily blood is a dark red opaque fluid due to the presence of the red blood corpuscles, but through the action of certain substances, such as water, ether, or chloroform, it may be rendered transparent. Blood so altered is said to be *laked*. The laking process is simply a liberation of the hæmoglobin from the stroma of the red blood corpuscle. Normal blood is alkaline in reaction[1] to litmus, the alkalinity being due principally to sodium carbonate and phosphate. The specific gravity of the blood of adults ordinarily varies between 1.045 and 1.075. It varies somewhat with the sex, the blood of males having a rather higher specific gravity than that of females of the same species. Under pathological conditions also the density of the blood may be very greatly altered. The freezing-point (Δ) of normal blood is about —0.56° C. Variations between —0.51° and 0.62° C. may be due entirely to dietary conditions, but if any marked variation is noted it can in most cases be traced to a disordered kidney function. The total amount of blood in the body has been variously estimated at from one-twelfth to one-fourteenth of the body weight. Perhaps 1/13.5 is the most satisfactory figure.

Among the most important constituents of blood plasma are the four protein bodies, *fibrinogen*, *nucleoprotein*, *serum globulin* (euglobulin and pseudo-globulin) and *serum albumin*. Plasma contains about 8.2 per cent of solids of which the protein constituents named above constitute approximately 84 per cent and the inorganic constituents (mainly chlorides, phosphates and carbonates) approximately 10 per cent. Among the inorganic constituents sodium chloride predominates. To prevent coagulation, blood plasma is ordinarily studied in the form

[1] Recently it has been shown by physico-chemical methods that the blood is in reality neutral in reaction.

of an oxalated or salted plasma. The former may be obtained by allowing the blood to flow from an opened artery into an equal volume of 0.2 per cent ammonium oxalate solution, whereas in the preparation of a salted plasma 10 per cent sodium chloride solution may be used as the diluting fluid.

Fibrinogen is perhaps the most important of the protein constituents of the plasma. It is also found in lymph and chyle as well as in certain exudates and transudates. Fibrinogen possesses the general properties of the globulins, but differs from serum globulin in being precipitated upon half-saturation with sodium chloride. In the process of coagulation of the blood the fibrinogen is transformed into fibrin. This fibrin is one of the principal constituents of the ordinary blood clot.

The nucleoprotein of blood possesses many of the characteristics of serum globulin. In common with this body it is easily soluble in sodium chloride, and is completely precipitated from its solutions upon saturation with magnesium sulphate. It is much less soluble in dilute acetic acid than serum globulin, and its solutions coagulate at 65°–69° C.

The body formerly called serum globulin is probably not an individual substance. Recent investigations seem to indicate that it may be resolved into two individual bodies called *euglobulin* and *pseudoglobulin*. The euglobulin is practically insoluble in water and may be precipitated in the presence of 28–36 per cent of saturated ammonium sulphate solution. The pseudoglobulin, on the contrary, is soluble in water and is only precipitated by ammonium sulphate in the presence of from 36 to 44 per cent of saturated ammonium sulphate solution.

In common with serum globulin the body known as serum albumin seems also to consist of more than a single individual substance. The so-called serum albumin may be separated into at least two distinct bodies, one capable of crystallization, the other an amorphous body. The solution of either of these bodies in water gives the ordinary albumin reactions. The coagulation temperature of the serum albumin mixture as it occurs in serum or plasma varies from 70° to 85° C. according to the reaction of the solution and its content of inorganic material. Serum albumin differs from egg albumin in being more lævorotatory, in being rendered less insoluble by alcohol, and in the fact that when precipitated by hydrochloric acid it is more easily soluble in an excess of the reagent.

When blood coagulates and the usual clot forms, a light yellow fluid exudes. This is blood *serum*. It differs from blood plasma

in containing a large amount of *fibrin ferment*, a body of great importance in the coagulation of the blood, and also in possessing a lower protein content. The protein material present in plasma and not found in serum is the fibrinogen which is transformed into fibrin in the process of coagulation and removed. The specific gravity of the serum of human blood varies between 1.026 and 1.032. If blood be drawn into a vessel and allowed to remain without stirring or agitation of any sort the major portion of the red corpuscles will sink away from the upper surface, causing this portion of the clot to assume a lighter color due to the predominance of leucocytes. This light colored portion of the clot is called the "buffy coat."

Beside the protein constituents already mentioned, other bodies which are found in both the plasma and serum are the following: *Sugar* (dextrose), *fat*, *enzymes*, *lecithin*, *cholesterol* and its esters, *gases*, *coloring-matter* (lutein or lipochrome) and *mineral substances*. In addition to these bodies the following substances have been detected in normal human blood: *Creatine*, *carbamic acid*, *hippuric acid*, *paralactic acid*, *urea* and *uric acid* (*urates*). Some of the *pathological constituents* of blood are *proteoses*, *leucine*, *tyrosine* and other amino acids, *biliary constituents* and *purine bodies*.

There has recently been considerable controversy regarding the form of the erythrocytes or red blood corpuscles of human blood. It is claimed by some investigators that the cells are *bell-shaped* or *cup-shaped*. As the erythrocytes occur normally *in the circulation*, however, they are probably thin, non-nucleated, biconcave discs. When examined singly under the microscope, they possess a pale greenish-yellow color (see Plate IV, opposite), whereas when grouped in large masses a reddish tint is noted.

The blood of most mammals contains erythrocytes similar in form to those of human blood. In the blood of birds, fishes, amphibians and reptiles the erythrocytes are ordinarily more or less elliptical, biconvex and possess a nucleus. The erythrocytes vary in size with the different animals. The average diameter of the erythrocytes of blood from various species is given in the following table:[1]

Elephant	$\frac{1}{2738}$ of an inch.
Guinea-pig	$\frac{1}{3223}$ of an inch.
Man	$\frac{1}{3250}$ of an inch.
Monkey	$\frac{1}{3882}$ of an inch.
Dog	$\frac{1}{3561}$ of an inch.
Rat	$\frac{1}{3632}$ of an inch.

[1] Wormley's Micro-Chemistry of Poisons, second edition, p. 733.

PLATE IV.

NORMAL ERYTHROCYTES AND LEUCOCYTES.

Rabbit	$\frac{1}{3633}$ of an inch.
Mouse	$\frac{1}{3743}$ of an inch.
Lion	$\frac{1}{4143}$ of an inch.
Ox	$\frac{1}{4219}$ of an inch.
Horse	$\frac{1}{4243}$ of an inch.
Pig	$\frac{1}{4268}$ of an inch.
Cat	$\frac{1}{4372}$ of an inch.
Sheep	$\frac{1}{4912}$ of an inch.
Goat	$\frac{1}{6189}$ of an inch.
Musk-deer	$\frac{1}{12525}$ of an inch.

The erythrocytes from whatever source obtained, consist essentially of two parts, the *stroma* or protoplasmic tissue and its enclosed pigment, *hæmoglobin*. For human blood the number of erythrocytes present in the fluid as obtained from well-developed males in good physical condition is about 5,500,000 per cubic millimeter.[1] The normal content of the blood of adult females is from 4,000,000 to 4,500,000 per cubic millimeter. The number of erythrocytes varies greatly under different conditions. For instance the number may be increased after the transfusion of blood of the same species of animal; by residing in a high altitude; or as a result of strenuous physical exercise continued over a short period of time. An increase is also noted in starvation; after partaking of food; after cold or hot baths; after massage, as well as after the administration of certain drugs and accompanying certain diseases, such as cholera, diarrhœa, dysentery and yellow atrophy of the liver. A decrease in the number occurs in the different forms of anæmia. The number has been known to increase to 7,040,000 per cubic millimeter as a result of physical exercise, while 11,000,000 per cubic millimeter have been noted in cases of polycythæmia and increases nearly as great in cyanosis. The number has been known to decrease to 500,000 per cubic millimeter or lower in pernicious anæmia.

Oxyhæmoglobin, the coloring matter of the blood, is a conjugated protein. Through treatment with hydrochloric acid it may be split into a protein body called *globin*, and *hæmochromogen*, an iron-containing pigment. The latter body is rapidly transformed into *hæmatin* in the presence of oxygen, and this in turn gives place to hæmatin-hydrochloride or *hæmin* (Figs. 58 and 59, page 194). The pigment of arterial blood is for the most part loosely combined with oxygen and is termed *oxy*hæmoglobin, whereas the pigment of venous blood is principally hæmoglobin (so-called *reduced* hæmoglobin). Oxyhæmoglobin

[1] This statement is based upon observations made upon the blood of athletes in training. It is generally stated in text-books that the blood of males contains about 5,000,000 per cubic millimeter.

FIG. 50.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE GUINEA-PIG.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

FIG. 51.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE RAT.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

FIG. 52.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE HORSE.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

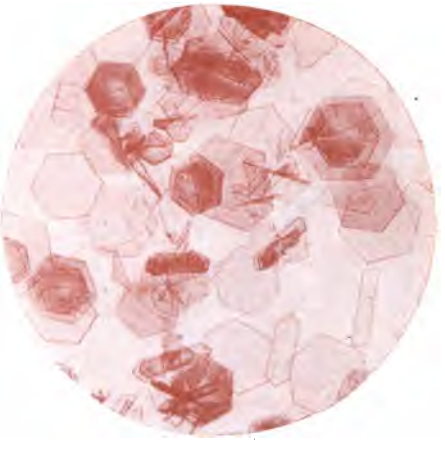


FIG. 53.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE SQUIRREL.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

FIG. 54.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE DOG.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

FIG. 55.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE CAT.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

FIG. 56.—OXYHÆMOGLOBIN CRYSTALS FROM BLOOD OF THE NECTURUS.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.[1]

is the oxygen-carrier of the body and belongs to the class of bodies known as respiratory pigments. It is held within the stroma of the erythrocyte. The reduction of oxyhæmoglobin to form hæmoglobin (so-called *reduced* hæmoglobin) occurs in the capillaries. Oxyhæmoglobin may be crystallized and a specific form of crystal obtained from the blood of each individual species (see Figs. 50 to 56, pages 182 to 185). This fact seems to indicate that there are many varieties of oxyhæmoglobin. The interesting findings of Reichert and Brown are of great value in this connection. These investigators prepared oxyhæmoglobin crystals from over *one hundred* species of animal and subsequently studied the characteristics of the crystals very minutely from the standpoint of crystallography. Their findings may prove of importance from the standpoint of heredity and the origin of species. They emphasize the following facts:

1. Crystals from all species of a certain genus have certain characteristics in general. Crystals from different genera, however, exhibit marked differences in *system, axial ratios*, etc.

2. Crystals of different species of a genus may generally be differentiated by difference in the angles.

3. The oxyhæmoglobin of some species crystallizes in several types of crystals in the same preparation. Generally the crystals first formed belong to a system of a lower grade of symmetry than those formed

[1] The micro-photographs of oxyhæmoglobin (see pages 182–185) and hæmin (see page 194) are reproduced through the courtesy of Professors E. T. Reichert and Amos P. Brown, of the University of Pennsylvania, who are investigating the crystalline forms of biochemic substances.

later. When such different types of crystals occur they may be arranged in isomorphous series.

4. Certain definite angles recur in the crystals from the blood of various species of animal, although the zoölogical connection may be remote and the crystals belong to different systems.

5. The constant recurrence of certain types of "twinning" in all the crystalline forms was observed.

6. Differences have been observed in the crystalline form of oxyhæmoglobin and hæmoglobin from the blood of the same species in certain cases.

The following bodies may be derived from hæmoglobin, and each possesses a specific spectrum which serves as an aid in its detection and identification: Oxyhæmoglobin, methæmoglobin, carbon-monoxide hæmoglobin, nitric-oxide hæmoglobin, hæmochromogen, hæmatin, acid-hæmatin, alkali-hæmatin and hæmatoporphyrin (see Absorption Spectra, Plates I and II).

The white corpuscles (or leucocytes) of human blood differ from the red corpuscles (or erythrocytes) in many particulars, such as being somewhat larger in size, in containing at least a single nucleus and in possessing amœboid movement (see Plate IV, opposite page 180). They are typical animal cells and therefore contain the following bodies which are customarily present in such cells: *Proteins, fats, carbohydrates, lecithin, cholesterol, inorganic salts* and *water*. The normal number of leucocytes in human blood varies between 5,000 and 10,000 per cubic millimeter. The ratio between the leucocytes and erythrocytes is about 1:350–500. A *leucocytosis* is said to exist when the number of leucocytes is increased for any reason. Leucocytoses may be divided into two general classes, the *physiological* and the *pathological*. Under the physiological form would be classed those leucocytoses accompanying pregnancy, parturition and digestion, as well as those due to mechanical and thermal influences. The leucocytoses spoken of as pathological are the inflammatory, infectious, post-hæmorrhagic, toxic and experimental forms as well as the type of leucocytosis which accompanies malignant disease.

The blood plates (platelets or plaques) are round or oval, colorless discs which possess a diameter about one-third as great as that of the erythrocytes. Upon treatment with certain reagents, *e. g.*, artificial gastric juice, they may be separated into a homogeneous, non-refractive portion and a granular, refractive portion. The blood plates are probably associated in some way with the coagulation of the blood. This relationship is not well understood at present.

The hæmoconein or so-called "blood dust" is made up of round granules which usually have a diameter somewhat less than one micron. The serum of normal as well as of pathological blood contains these granules. They were first described by Müller to whom they appeared as highly refractile granules possessed of Brownian movement. The "blood dust" is apparently not concerned with the coagulation of the blood. The granules are insoluble in alcohol, ether and acetic acid and are not blackened by osmic acid. According to Müller, the granules making up the so-called "blood dust" constitute a new organized constituent of the blood, whereas other investigators believe them to be merely free granules from certain of the forms of leucocytes. In common with blood plates the "blood dust" possesses no clinical significance.

The processes involved in the coagulation of the blood are not fully understood. Several theories have been advanced and each has its adherents. The theory which appears to be fully as firmly founded upon experimental evidence as any is the following: Blood contains a zymogen called *prothrombin* which combines with the calcium salts present to form an enzyme known as *thrombin* or *fibrin-ferment.* When freshly drawn blood comes in contact with the air the fibrin-ferment at once acts upon the fibrinogen present and gives rise to the formation of *fibrin.* This fibrin forms in shreds throughout the blood mass and, holding the form elements of the blood within its meshes, serves to produce the typical *blood clot.* The fibrin shreds gradually contract, the whole clot assumes a jelly-like appearance and the yellowish serum exudes. If, immediately upon the withdrawal of blood from the body, the fluid be rapidly stirred or thoroughly "whipped" with a bundle of coarse strings, twigs or a specially constructed beater, the fibrin shreds will not form in a network throughout the blood mass but instead will cling to the device used in beating. In this way the fibrin may be removed and the remaining fluid is termed *defibrinated* blood. The above theory of the coagulation of the blood may be stated briefly as follows:

I. Prothrombin + Calcium Salts = Thrombin (or Fibrin-ferment).

II. Thrombin (or Fibrin-ferment) + Fibrinogen = Fibrin.

Among the medico-legal tests for blood are the following: (1) Microscopical identification of the erythrocytes, (2) spectroscopic identification of blood solutions, (3) the guaiac test, (4) the benzidine reaction, (5) preparation of hæmin crystals. Of these five tests the two last named are generally considered to be the most satisfactory. They give equally reliable results with fresh blood and with blood from

clots or stains of long standing, provided the latter have not been exposed to a high temperature, or to the rays of the sun for a long period. The technique of the tests is simple and the formation of the dark brown or chocolate colored crystals of hæmin or the production of the green or blue color with benzidine is indisputable proof of the presence of blood in the fluid, clot or stain examined. The weak point of the tests, medico-legally, lies in the fact that they do not differentiate between human blood and that of certain other species of animal.

The guaiac test (see page 191), although generally considered less accurate than the hæmin test, is really a more delicate test than the hæmin test if properly performed. One of the most common mistakes in the manipulation of this test is the use of a guaiac solution which is too concentrated and which, when brought into contact with the aqueous blood solution, causes the separation of a voluminous precipitate of a resinous material which may obscure the blue coloration: this is particularly true of the test when used for the examination of blood stains. A solution of guaiac made by dissolving 1 gram of the resin in 60 c.c. of 95 per cent alcohol is very satisfactory for general use. The test is frequently objected to upon the ground that various other substances, *e. g.*, milk, pus, saliva, etc., respond to the test and that it cannot therefore be considered a specific test for blood and is of value only in a negative sense. We have demonstrated to our own satisfaction, however, that milk many times gives the blue color upon the addition of an alcoholic solution of guaiac resin without the addition of hydrogen peroxide or old turpentine. Buckmaster has very recently advocated the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiac resin. He claims that he was able to produce the blue color upon the addition of the guaiaconic acid to milk *only* when the sample of milk tested was brought from the country in *sterile bottles*, and further, that no sample of London milk which he examined responded to the test. In the application of the guaiac test to the detection of blood, he states that he was able to detect *laked blood* when present in the ratio 1:5,000,000 and *unlaked blood* when present in the ratio 1:1,000,000. This author considers the guaiac test to be far more trustworthy than is generally believed.

Up to within very recent times it has been impossible to make an absolute differentiation of human blood. Recently, however, the so-called "biological" blood test has made such a differentiation possible. This test, known as the Bordet reaction, is founded upon the fact that the blood serum of an animal into which has been injected the blood of another animal of different species develops the property of

agglutinating and dissolving erythrocytes *similar to those injected*, but exerts this influence upon the blood from *no other species*. The antiserum used in this test is prepared by injecting rabbits with 5–10 c.c. of human defibrinated blood, at intervals of about four days until a total of between 50 and 80 c.c. has been injected. After a lapse of one or two weeks the animal is bled, the serum collected, placed in sterile tubes and preserved for use as needed. In examining any specific solution for human blood it is simply necessary to combine the antiserum and the solution under examination in the proportion of 1:100 and place the mixture at 37° C. If human blood is present in the solution a turbidity will be noted and this will change within three hours to a distinctly flocculent precipitate. This antiserum will react thus with no other known substance.

Experiments on Blood.

I. Defibrinated Ox-blood.

1. **Reaction.**—Moisten red and blue litmus papers with 10 per cent sodium chloride solution and test the reaction of the defibrinated blood.

2. **Microscopical Examination.**—Examine a drop of defibrinated blood under the microscope. Compare the objects you observe with Plate IV, opposite page 180. Repeat the test with a drop of your own blood.

3. **Specific Gravity.**—Determine the specific gravity of defibrinated blood by means of an ordinary specific gravity spindle. Compare this result with the specific gravity as determined by Hammerschlag's method in the next experiment.

4. **Specific Gravity by Hammerschlag's Method.**—Fill an ordinary urinometer cylinder about one-half full of a mixture of chloroform and benzene, having a specific gravity of approximately 1.050. Into this mixture allow a drop of the blood under examination to fall from a pipette or directly from the finger in case fresh blood is being examined. Care must be taken not to use too large a drop of blood and to keep the drop from coming in contact with the walls of the cylinder. If the blood drop sinks to the bottom of the vessel, thus showing it to be of higher specific gravity than the surrounding fluid, add chloroform until the blood drop remains suspended in the mixture. Stir carefully with a glass rod after adding the chloroform. If the blood drop rises to the surface upon being introduced into the mixture, thus showing it to be of lower specific gravity than the surrounding

fluid, add benzene until the blood drop remains suspended in the mixture. Stir with a glass rod after the benzene is added. After the blood drop has been brought to a suspended position in the mixture by means of one or more additions of chloroform and benzene this final mixture should be filtered through muslin and its specific gravity accurately determined. What is the specific gravity of the blood under examination?

5. **Tests for Various Constituents.**—Place 10 c.c. of defibrinated blood in an evaporating dish, dilute with 100 c.c. of water and heat to boiling. Is there any coagulation, and if so what bodies form the coagulum? At the boiling-point acidulate slightly with dilute acetic acid. Filter. The filtrate should be clear and the coagulum dark brown. Reserve this coagulum. What body gives the coagulum this color? Evaporate the filtrate to about 25 c.c., filtering off any precipitate which may form in the process. Make the following tests upon the filtrate:

(*a*) *Fehling's Test.*—Test for sugar according to directions given on page 27.

(*b*) *Chlorides.*—To a small amount of the filtrate in a test-tube add a few drops of nitric acid and a little argentic nitrate. In the presence of chloride, a white precipitate of argentic chloride will form.

(*c*) *Phosphates.*—Test for phosphates by nitric acid and molybdic solution according to directions given on page 56.

(*d*) *Proteose and Peptone.*—Test a small amount of the solution for proteose and peptone by saturating with ammonium sulphate according to directions given on page 112.

(*e*) *Crystallization of Sodium Chloride.*—Place the remainder of the filtrate in a watch glass and evaporate it on a water-bath. Examine the crystals under the microscope and compare them with those in Fig. 60, page 196.

6. **Test for Iron.**—Incinerate a small portion of the coagulum from the last experiment (5) in a porcelain crucible. Cool, dissolve the residue in dilute hydrochloric acid and test for iron by potassium ferrocyanide or ammonium thiocyanate. Which of the constituents of the blood contains the iron?

7. **Laky Blood.**—Note the opacity of ordinary defibrinated blood. Place a few cubic centimeters of this blood in a test-tube and add water, a little at a time, until the blood is rendered transparent. It is now *laky* blood. How does the water act in causing this transparency? Examine a drop of laky blood under the microscope. How does its microscopical appearance differ from that of

unaltered blood? What other agents may be used to render blood laky?

8. **Osmotic Pressure.**—Place a few cubic centimeters of blood in each of three test-tubes. Lake the blood in the first tube according to directions given in the last experiment (7): add an equal volume of *isotonic* (0.9 per cent) sodium chloride to the blood in the second tube, and an equal volume of 10 per cent sodium chloride to the blood in the third tube. Mix thoroughly by shaking and after a few moments examine a drop from each of the three tubes under the microscope (see Figs. 57 and 115, pages 191 and 354). What do you find and what is your explanation from the standpoint of osmotic pressure?

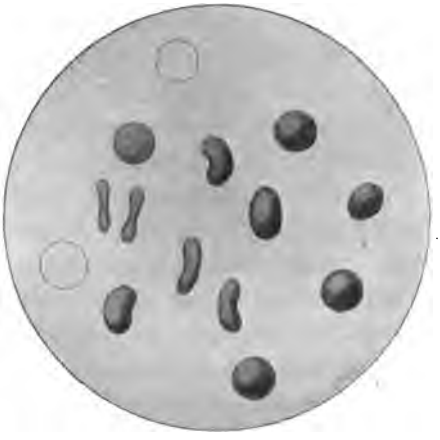


FIG. 57.—EFFECT OF WATER ON ERYTHROCYTES.

9. **Agglutination.**—To about 5 c.c. of a dilute sodium chloride solution of ricin[1] in a test-tube add about one-half cubic centimeter of defibrinated blood and shake the mixture thoroughly. Allow the tube to stand about 15 minutes and examine a drop of the contents under the microscope. Note the "clumping" or "agglutination" of the erythrocytes, and contrast this phenomena with the appearance of normal blood as just examined in Experiment 8.

10. **Diffusion of Hæmoglobin.**—Prepare some laky blood, thus liberating the hæmoglobin from the erythrocytes. Test the diffusion of the hæmoglobin by preparing a dialyzer like one of the models shown in Fig. 1, page 25. How does hæmoglobin differ from other well-known crystallizable bodies?

11. **Guaiac Test.**—To 5 c.c. of water in a test-tube add two drops of blood. By means of a pipette drop an alcoholic solution

[1] A protein constituent of the castor bean.

of guaiac (strength about 1: 60)[1] into the resulting mixture until a turbidity is observed and add old turpentine or hydrogen peroxide, drop by drop, until a blue color is obtained. Do any other substances respond in a similar manner to this test? Is a positive guaiac test a sure indication of the presence of blood?

12. **Schumm's Modification of the Guaiac Test.**—To about 5 c.c. of the solution under examination[2] in a test-tube add about ten drops of freshly prepared alcoholic solution of guaiac. Agitate the tube gently, add about 20 drops of old turpentine, subject the tube to a thorough shaking and permit it to stand for about 2–3 minutes. A blue color indicates the presence of blood in the solution under examination. In case there is insufficient blood to yield a blue color under these conditions, a few c.c. of alcohol should be added and the tube gently shaken, whereupon a blue coloration will appear in the upper alcohol-turpentine layer.

A control test should always be made, using water in place of the solution under examination. In the detection of very minute traces of blood only 3–5 drops of the guaiac solution should be employed.

13. **Adler's Benzidine Reaction.**—This is one of the most delicate of the reactions for the detection of blood. Different benzidine preparations vary greatly in their sensitiveness, however. Inasmuch as benzidine solutions change readily upon contact with light it is essential that they be kept in a dark place. The test is performed as follows: To a saturated solution of benzidine in alcohol or glacial acetic acid add an equal volume of 3 per cent hydrogen peroxide and one c.c. of the solution under examination. If the mixture is not already acid render it so with acetic acid, and note the appearance of a green or blue color. A control test should be made substituting water for the solution under examination. The sensitiveness of the benzidine reaction is greater when applied to aqueous solutions than when applied to the urine. According to Ascarelli[3] the benzidine reaction serves to detect blood when present in a dilution of 1: 300,000. Walter[4] has also recently shown the test to be very delicate and claims it to be more satisfactory than the guaiac test.

14. **Hæmin Test.**—(*a*) *Teichmann's Method.*—Place a *very small* drop of blood on a microscopic slide, add a minute grain of sodium

[1] Buckmaster advises the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiac resin.

[2] Alkaline solutions should be made slightly acid with acetic acid, as the blue end-reaction is very sensitive to alkali.

[3] Ascarelli: *Il policlin sez. prat.*, 1909.

[4] Walter: *Deut. med. Woch.*, 36, p. 309.

chloride[1] and *carefully* evaporate to *dryness* over a *low flame*. Put a cover glass in place, run underneath it a drop of *glacial* acetic acid and *warm gently* until the formation of gas bubbles is noted. Add another drop of glacial acetic acid, cool the preparation, examine under the microscope and compare the crystals with those shown in Figs. 58 and 59, page 194. The hæmin crystals result from the decomposition of the hæmoglobin of the blood. What are the steps involved in this process? The hæmin crystals are also called Teichmann's crystals. Is this an *absolute* test for blood? Is it possible to differentiate between human blood and the blood of other species by means of the hæmin test?

(*b*) *Atkinson and Kendall's Method.*—Introduce a small amount of the solution under examination into a tube closed at one end, add sodium chloride and glacial acetic acid as in Teichmann's method,[2] fuse or tightly plug the open end of the tube and heat for fifteen minutes in a boiling water-bath.[3] Remove the tube and permit it to cool to room temperature spontaneously. When the tube has cooled, break it open, transfer the contents to a watch glass or small evaporating dish and concentrate on a water-bath until the volume of the fluid in the watch glass or dish has been reduced to a few drops. Transfer a drop of this fluid to a slide, cover with a cover slip, allow the slide to stand for a few minutes and examine it under a microscope. Compare the crystals with those shown in Figs. 58 and 59, page 194. In case crystals of sodium chloride (see Fig. 60, page 196) obstruct the view of the hæmin crystals, dissolve the sodium chloride crystals by running a drop of water under the cover slip.

(*c*) *v. Zeynek and Nencki's Method.*—To 10 c.c. of defibrinated blood add acetone until no more precipitate forms. Filter off the precipitated protein and extract it with 10 c.c. of acetone made acid with 2–3 drops of hydrochloric acid. Place a drop of the resulting colored extract on a slide, immediately place a coverglass in position and examine under the microscope. Upon the evaporation of the acetone, crystals of hæmin will form. Larger crystals may be obtained by evaporating the acetone extract about one-half, transferring it to a stoppered vessel and allowing it to remain overnight.

(*d*) *Schalfijew's Method.*—Place 20 c.c. of glacial acetic acid in a small beaker and heat to 80° C. Add 5 c.c. of strained defibrinated blood, again bring the temperature to 80° C., remove the flame and

[1] Buckmaster considers the use of potassium chloride preferable.
[2] Care should be taken not to add too great an excess of these reagents.
[3] This process insures constancy of temperature and strength of reagents.

FIG. 58.—HÆMIN CRYSTALS FROM HUMAN BLOOD.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

FIG. 59.—HÆMIN CRYSTALS FROM SHEEP BLOOD.
Reproduced from a micro-photograph furnished by Prof. E. T. Reichert, of the University of Pennsylvania.

allow the mixture to cool. Examine the crystals under the microscope and compare them with those reproduced in Figs. 58 and 59, page 194.

15. **Catalytic Action.**—To about 10 drops of blood in a test-tube add twice the volume of hydrogen peroxide, without shaking. The mixture foams. What is the cause of this phenomenon?

16. **Preparation of Hæmatin.**—Place 100 c.c. of *laked* blood in a beaker and add 95 per cent alcohol until precipitation ceases. What bodies are precipitated? Transfer the precipitate to a flask and boil with 95 per cent alcohol previously acidulated with sulphuric acid. Through the action of the acid the hæmoglobin is split into hæmatin and a protein body called globin. Later the "sulphuric acid ester of hæmatin" is formed, which is soluble in the alcohol. Continue heating until the precipitate is no longer colored, then filter. Partly saturate the filtrate with sodium chloride and warm. In this process the "hydrochloric acid ester of hæmatin" is formed. Filter and dissolve on the filter paper by sodium carbonate. Save this alkaline solution of hæmatin and make a spectroscopic examination later after becoming familiar with the use of the spectroscope. How does the spectrum of oxyhæmoglobin differ from that of the derived *alkali hæmatin?*

17. **Variation in Size of Erythrocytes.**—Prepare two small funnels with filter papers such as are used in quantitative analysis. Moisten each paper with normal (isotonic) salt solution. Into one funnel introduce a small amount of defibrinated ox blood and into the other funnel allow blood to drop directly from a decapitated frog. Note that the filtrate from the ox blood is colored whereas that from the frog blood is colorless. What deduction do you make regarding the relative size of the erythrocytes in ox and frog blood? Does either filtrate clot? Why?

II. Blood Serum.

1. **Coagulation Temperature.**—Place 5 c.c. of undiluted serum in a test-tube and determine its temperature of coagulation according to the method described on page 98. Note the temperature at which a cloudiness occurs as well as the temperature at which coagulation is complete.

2. **Precipitation by Alcohol.**—To 5 c.c. of serum in a test-tube add twice the amount of 95 per cent alcohol and thoroughly mix by shaking. What is this precipitate? Make a confirmatory test. Test the alcoholic filtrate for protein. Explain the result.

3. **Proteins of Blood Serum.**—Place about 20 c.c. of undiluted serum in a small evaporating dish, heat to boiling, and at the boiling-point acidify slightly with dilute acetic acid. Of what does this coagulum consist? Filter off the coagulum (reserve the filtrate) and test it as follows:

(*a*) *Millon's Reaction.*—Make the test according to directions given on page 88.

(*b*) *Hopkins-Cole Reaction.*—Make the test according to directions given on page 89.

FIG. 60.—SODIUM CHLORIDE.

4. **Sugar in Serum.**—Test a little of the filtrate from Experiment 3 by Fehling's test. What do you conclude?

5. **Detection of Sodium Chloride.**—(*a*) Test a little of the filtrate from Experiment 3 for chlorides, by the use of nitric acid and argentic nitrate. (*b*) Evaporate 5 c.c. of the filtrate from Experiment 3 in a watch glass on a water-bath. Examine the crystals and compare them with those reproduced in Fig. 60, above.

6. **Separation of Serum Globulin and Serum Albumin.**—Place 10 c.c. of blood serum in a small beaker and saturate with magnesium sulphate. What is this precipitate? Filter it off and acidify the filtrate slightly with acetic acid. What is this second precipitate? Filter this precipitate off and test the filtrate by the biuret test. What do you conclude?

III. Blood Plasma.

1. **Preparation of Oxalated Plasma.**—Allow arterial blood to run into an equal volume of 0.2 per cent ammonium oxalate solution.

2. **Preparation of Fibrinogen.**—To 25 c.c. of oxalated plasma add an equal volume of saturated sodium chloride solution. Note the precipitation of fibrinogen. Filter off the precipitate (reserve the filtrate) and test it by a protein color test (see page 88).

3. **Effect of Calcium Salts.**—Place a small amount of oxalated plasma in a test-tube and add a few drops of a 2 per cent calcium chloride solution. What occurs? Explain it.

4. **Preparation of Salted Plasma.**—Allow arterial blood to run into an equal volume of a saturated solution of sodium sulphate or a 10 per cent solution of sodium chloride. Keep the mixture in a cold place for about twenty-four hours.

5. **Effect of Dilution.**—Place a few drops of salted plasma in a test-tube and dilute it with 10–15 volumes of water. What do you observe? Explain it.

6. **Crystallization of Oxyhæmoglobin.**—*Reichert's Method.*—Add to 5 c.c. of the blood of the dog, horse, guinea-pig, or rat, before or after laking, or defibrinating, from 1 to 5 per cent of ammonium oxalate *in substance.* Place a drop of this oxalated blood on a slide and examine under the microscope. The crystals of oxyhæmoglobin will be seen to form at once near the margin of the drop, and in a few minutes the entire drop may be a solid mass of crystals. Compare the crystals with those shown in Figs. 50 to 56, pages 182 to 185.

IV. Fibrin.

1. **Preparation of Fibrin.**—Allow blood to flow directly from the animal into a vessel and rapidly *whip* it by means of a bundle of twigs, a mass of strong cords, or a specially constructed beater. If a pure fibrin is desired it is not best to attempt to manipulate a large volume of blood at one time. After the fibrin has been collected it should be freed from any adhering blood clots and washed in water to remove further traces of blood. The pure product should be very light in color. It may be preserved under glycerol, dilute alcohol, or chloroform water.

2. **Solubility.**—Try the solubility of small shreds of freshly prepared fibrin in the usual solvents.

3. **Millon's Reaction.**—Make the test according to directions given on page 88.

4. **Hopkins-Cole Reaction.**—Make the test according to directions given on page 89.

5. **Biuret Test.**—Make the test according to directions given on page 90.

V. Detection of Blood in Stains on Cloth, etc.

1. **Identification of Corpuscles.**—If the stain under examination is on cloth a portion should be extracted with a few drops of glycerol or normal (0.9 per cent) sodium chloride solution. A drop of this solution should then be examined under the microscope to determine if corpuscles are present.

2. **Tests on Aqueous Extract.**—A second portion of the stain should be extracted with a small amount of water and the following tests made upon the aqueous extract:

(*a*) *Hæmochromogen.*—Make a small amount of the extract alkaline by potassium hydroxide or sodium hydroxide, and heat until a brownish-green color results. Cool and add a few drops of ammonium sulphide or Stokes' reagent (see page 199) and make a spectroscopic examination. Compare the spectrum with that of hæmochromogen (see Absorption Spectra, Plate II).

(*b*) *Hæmin Test.*—Make this test upon a small drop of the aqueous extract according to the directions given on page 192.

(*c*) *Guaiac Test.*—Make this test on the aqueous extract according to the directions given on page 191. The guaiac solution may also be applied directly to the stain without previous extraction in the following manner: Moisten the stain with water, and after allowing it to stand several minutes, add an alcoholic solution of guaiac (strength about 1:60) and a little hydrogen peroxide or old turpentine. The customary blue color will be observed in the presence of blood.

(*d*) *Benzidine Reaction.*—Make this test according to directions given on p. 192.

(*e*) *Acid Hæmatin.*—If the stain fails to dissolve in water extract with acid alcohol and examine the spectrum for absorption bands of acid hæmatin (see Absorption Spectra, Plate II).

VI. Spectroscopic Examination of Blood.

(For Absorption Spectra see Plates I. and II.)

Either the *angular*-vision spectroscope (Figs. 62 and 63, page 200) or the *direct*-vision spectroscope (Fig. 61, page 199) may be used in making the spectroscopic examination of the blood. For a complete description of these instruments the student is referred to any standard text-book of physics.

1. **Oxyhæmoglobin.**—Examine dilute (1:50) defibrinated blood spectroscopically. Note the broad absorption-band between D and E. Continue the dilution until this single broad band gives place to

two narrow bands, the one nearer the D line being the narrower. These are the typical absorption-bands of oxyhæmoglobin obtained from dilute solutions of blood. Now dilute the blood *very freely* and note that the bands gradually become more narrow and, if the dilution is sufficiently great, they finally entirely disappear.

2. **Hæmoglobin (so-called *Reduced* Hæmoglobin).**—To blood which has been diluted sufficiently to show well defined oxyhæmoglobin absorption-bands add a small amount of Stokes' reagent.[1] The blood immediately changes in color from a bright red to violet-

FIG. 61.—DIRECT-VISION SPECTROSCOPE.

red. The oxyhæmoglobin has been reduced through the action of Stokes' reagent and hæmoglobin (so-called *reduced* hæmoglobin) has been formed. This has been brought about by the removal of some of the loosely combined oxygen from the oxyhæmoglobin. Examine this hæmoglobin spectroscopically. Note that in place of the two absorption bands of oxyhæmoglobin we now have a single broad band lying almost entirely between D and E. This is the typical spectrum of hæmoglobin. If the solution showing this spectrum be shaken in the air for a few moments it will again assume the bright red color of oxyhæmoglobin and show the characteristic spectrum of that pigment.

3. **Carbon Monoxide Hæmoglobin.**—The preparation of this pigment may be easily accomplished by passing ordinary illuminating gas[2] through defibrinated ox-blood. Blood thus treated assumes a brighter tint (carmine) than that imparted by oxyhæmoglobin. In very dilute solution oxyhæmoglobin appears yellowish-red whereas carbon monoxide hæmoglobin under the same conditions appears bluish-red. Examine the carbon monoxide hæmoglobin solution spectroscopically. Observe that the spectrum of this body resembles the spectrum of oxyhæmoglobin in showing two absorption-bands

[1] Stokes' reagent is a solution containing 2 per cent ferrous sulphate and 3 per cent tartaric acid. When needed for use a small amount should be placed in a test-tube and ammonium hydroxide added until the precipitate which forms on the first addition of the hydroxide has entirely dissolved. This produces *ammonium ferrotartrate* which is a reducing agent.

[2] The so-called water gas with which ordinary illuminating gas is diluted contains usually as much as 20 per cent of carbon monoxide (CO).

between D and E. The bands of carbon monoxide hæmoglobin, however, are somewhat nearer the violet end of the spectrum. Add some Stokes' reagent to the solution and again examine spectroscopically.

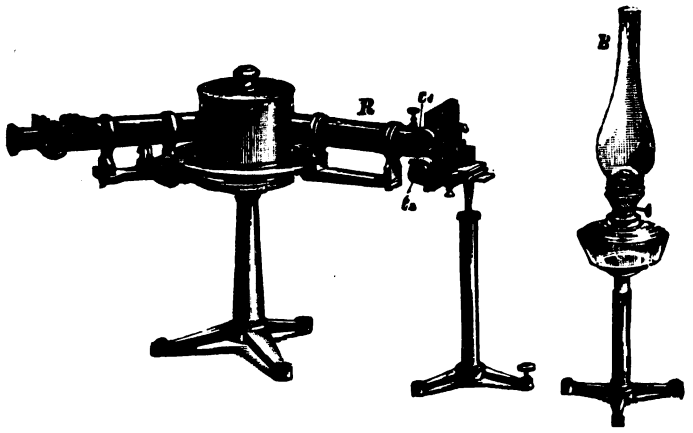


FIG. 62.—ANGULAR-VISION SPECTROSCOPE ARRANGED FOR ABSORPTION ANALYSIS.

Note that the position and intensity of the absorption-bands remain unaltered.

The following is a delicate *chemical* test for the detection of carbon monoxide hæmoglobin:

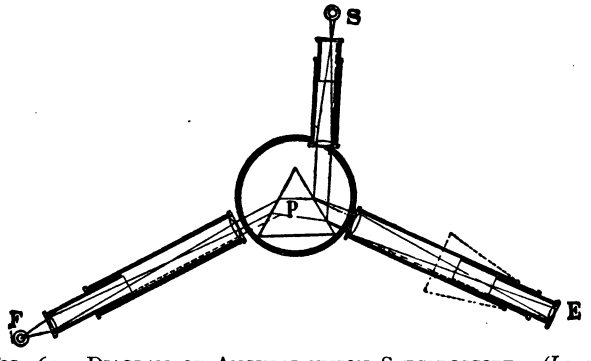


FIG. 63.—DIAGRAM OF ANGULAR-VISION SPECTROSCOPE. (*Long.*)

The white light *F* enters the collimator tube through a narrow slit and passes to the prism, *P*, which has the power of refracting and dispersing the light. The rays then pass to the double convex lens of the ocular tube and are deflected to the eye-piece *E*. The dotted lines show the magnified virtual image which is formed. The third tube contains a scale whose image is reflected into the ocular and shown with the spectrum. Between the light *F* and the collimator slit is placed a cell to hold the solution undergoing examination.

Tannin Test.—Divide the blood to be tested into two portions and dilute each with four volumes of distilled water. Place the diluted blood mixtures in two small flasks or large test-tubes and add 20

drops of a 10 per cent solution of potassium ferricyanide.[1] Allow both solutions to stand for a few minutes, then stopper the vessels and shake one vigorously for 10–15 minutes, occasionally removing the stopper to permit air to enter the vessel.[2] Add 5–10 drops of ammonium sulphide (yellow) and 10 c.c. of a 10 per cent solution of tannin to each flask. The contents of the shaken flask will soon exhibit the formation of a dirty olive green precipitate, whereas the flask which was not shaken and which, therefore, still contains carbon monoxide hæmoglobin, will exhibit a bright red precipitate, characteristic of carbon monoxide hæmoglobin. This test is more delicate than the spectroscopic test and serves to detect the presence of as low a content as 5 per cent of carbon monoxide hæmoglobin.

4. **Neutral Methæmoglobin.**—Dilute a little defibrinated blood (1 : 10) and add a few drops of a freshly prepared 10 per cent solution of potassium ferricyanide. Shake this mixture and observe that the bright red color of the blood is displaced by a brownish red. Now dilute a little of this solution and examine it spectroscopically. Note the single, very dark absorption-band lying to the left of D, and, if the dilution is sufficiently great, also observe the two rather faint bands lying between D and E in somewhat similar positions to those occupied by the absorption bands of oxyhæmoglobin. Add a few drops of Stokes' reagent to the methæmoglobin solution while it is in position before the spectroscope and note the immediate appearance of the oxyhæmoglobin spectrum which is quickly followed by that of hæmoglobin.

5. **Alkaline Methæmoglobin.**—Render a neutral solution of methæmoglobin, such as that used in the last experiment (4), slightly alkaline with a few drops of ammonia. The solution becomes redder in color, due to the formation of alkaline methæmoglobin and shows a spectrum different from that of the neutral body. In this case we have a band on either side of D, the one nearer the red end of the spectrum being much the fainter. A third band, darker than either of those mentioned, lies between D and E somewhat nearer E.

6. **Alkali Hæmatin.**—Observe the spectrum of the alkali hæmatin prepared in Experiment 16 on page 195. Also make a spectroscopic examination of a freshly prepared alkali hæmatin.[3] The

[1] This transforms the oxyhæmoglobin into methæmoglobin.

[2] This is done to free the blood from carbon monoxide hæmoglobin.

[3] Alkali hæmatin may be prepared by mixing one volume of a concentrated potassium hydroxide or sodium hydroxide solution and two volumes of dilute (1:5) defibrinated blood. This mixture should be heated gradually almost to boiling, then cooled and shaken for a few moments in the air before examination.

typical spectrum of alkali hæmatin shows a single absorption-band lying across D and mainly toward the red end of the spectrum.

7. **Reduced Alkali Hæmatin or Hæmochromogen.**—Dilute the alkali hæmatin solution used in the last experiment (6) to such an extent that it shows no absorption band. Now add a few drops of Stokes' reagent and note that the greenish-brown color of the alkali hæmatin solution is displaced by a bright red color. This is due to the formation of hæmochromogen or reduced alkali hæmatin. Examine this solution spectroscopically and observe the narrow, dark absorption-band lying midway between D and E. If the dilution is not too great a faint band may be observed in the green extending across E and b.

8. **Acid Hæmatin.**—To some defibrinated blood add half its volume of glacial acetic acid and an equal volume of ether. Mix thoroughly. The acidified ethereal solution of hæmatin rises to the top and may be poured off and used for the spectroscopic examination. If desired it may be diluted with acidified ether in the ratio of one part of glacial acetic acid to two parts of ether. A distinct absorption-band will be noted in the red between C and D and lying somewhat nearer C than the band in the methæmoglobin spectrum. Between D and F may be seen a rather indistinct broad band. Dilute the solution until this band resolves itself into two bands. Of these the more prominent is a broad, dark absorption-band lying in the green between b and F. The second, a narrow band of faint outline, lies in the light green to the red side of E. A fourth very faint band may be observed lying on the violet side of D.

9. **Acid Hæmatoporphyrin.**—To 5 c.c. of concentrated sulphuric acid in a test-tube add two drops of blood, mixing thoroughly by agitation after the addition of each drop. A wine-red solution is produced. Examine this solution spectroscopically. Acid hæmatoporphyrin gives a spectrum with an absorption-band on either side of D, the one nearer the red end of the spectrum being the narrower.

10. **Alkaline Hæmatoporphyrin.**—Introduce the acid hæmatoporphyrin solution just examined into an excess of distilled water. Cool the solution and add potassium hydroxide slowly until the reaction is but slightly acid. A colored precipitate forms which includes the principal portion of the hæmatoporphyrin. The presence of sodium acetate facilitates the formation of this precipitate. Filter off the precipitate and dissolve it in a small amount of dilute potassium hydroxide. Alkaline hæmatoporphyrin prepared in this way forms

a bright red solution and possesses four absorption-bands. The first is a very faint, narrow band in the red, midway between C and D; the second is a broader, darker band lying across D, principally to the violet side. The third absorption-band lies principally between D and E, extending for a short distance across E to the violet side, and the fourth band is broad and dark and lies between b and F. The first band mentioned is the faintest of the four and is the first to disappear when the solution is diluted.

VII. Instruments Used in the Clinical Examination of the Blood.

1. **Fleischl's Hæmometer** (Fig. 64, below).—This is an instrument used quite extensively clinically, for the quantitative determination of hæmoglobin. The instrument consists of a small cylinder which is provided with a fixed glass bottom and a movable glass cover, and which is divided, by means of a metal septum, into two compartments of equal capacity. This cylinder is supported in a vertical position by means of a mechanism which resembles the base and stage of an ordinary microscope. Underneath the stage is placed a colored glass wedge (see Fig. 66, p. 204), so arranged as to run immediately beneath the glass bottom of one of the compartments of the cylinder and ground in such a manner that each part of the wedge corresponds in color to a solution of hæmoglobin of some definite percentage. The glass wedge is held in a metal frame and may be moved backward or forward by means of a rack and pinion arrangement. A scale along the side of this frame indicates the percentage of the normal amount of hæmoglobin which each particular variation in the depth of color of the ground wedge represents, taking the normal hæmoglobin content as 100.[1] In a position corresponding to the position of the mirror on the ordinary microscope is attached a light-colored opaque plate which serves to reflect the light upward through the colored wedge and the cylinder to the eye of the observer.

FIG. 64.—FLEISCHL'S HÆMOMETER. (*Da Costa.*)

In making a determination of the percentage of hæmoglobin by this instrument the procedure is as follows: Fill each compartment

[1] The scale of the ordinary instrument is usually too high.

about three-fourths full of distilled water. Puncture the finger-tip or lobe of the ear of the subject by means of a sterile needle or scalpel and, as soon as a drop of blood appears, place one end of the capillary pipette (Fig. 65), which accompanies the instrument, against the drop and allow it to fill by capillary attraction. To prevent the blood from adhering to the exterior of the tube, and so render the determination inaccurate, it is customary to apply a very thin coating of mutton fat to the outer surface before using or to wrap the tube in a piece of oily chamois when not in use. As soon as the tube has been accurately filled with blood it should be dipped into the water of one of the compartments of the cylinder and all traces of the blood washed out with water by means of a small dropper which accompanies the instrument. If the blood is not well distributed throughout the compartment and does not form a homogeneous solution the contents of the compartment should be mixed thoroughly by means of the metal handle of the capillary measuring pipette. When this has been done each compartment should be completely filled with distilled water and the glass cover adjusted, care being taken that the contents of the two compartments do not mix. Now adjust the cylinder so that the compartment containing the pure distilled water is immediately above the colored glass wedge. By means of the rack and pinion arrangement manipulate the colored wedge until a portion of it is found which corresponds in color with the diluted blood. When this agreement in color has been secured the point on the scale corresponding to this particular color should be read and the actual percentage of hæmoglobin computed. For instance, if the scale reading is 90 it means that the blood under examination contains 90 per cent of the normal quantity of hæmoglobin, *i. e.*, 90 per cent of 14 per cent.

FIG. 65.—PIPETTE OF FLEISCHL'S HÆMOMETER.

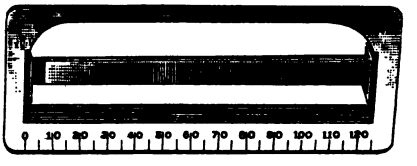


FIG. 66.—COLORED GLASS WEDGE OF FLEISCHL'S HÆMOMETER. (*Da Costa.*)

2. **Fleischl-Miescher Hæmometer.**—The apparatus of Fleischl has recently been modified by Miescher. If all precautions are taken, the margin of error in the absolute quantity of hæmoglobin determined by this instrument does not exceed 0.15–0.22 per cent by weight of the blood. Detailed directions for the manipulation of the Fleischl-

Miescher hæmometer accompany the instrument. In brief Miescher modified the instrument as follows: (1) The scale of each instrument is supplied with a caliber table of *absolute* hæmoglobin values, expressed in milligrams: the scale of Fleischl's hæmometer shows the percentage of hæmoglobin in relation to an average selected somewhat arbitrarily. Thus many errors arising from the irregular coloring of the glass wedge of the older apparatus are avoided in the instrument as modified. (2) Each instrument is accompanied by a measuring pipette (melangeur) which allows of a more accurate measurement of the blood than was possible with the capillary tubes of the older apparatus. (3) With the aid of the measuring pipette mentioned above blood of varying degrees of concentration may be compared. In this way the individual examinations are controlled and a check upon the accuracy of the graduation in the color of the glass wedge is also afforded. This wedge is much more evenly and accurately colored than in the unmodified apparatus of Fleischl. (4) Before reading the percentage as indicated by the scale, the chamber is covered with a glass and a diaphragm which sharply define the field on all sides without the formation of a meniscus.

The measuring pipette is constructed essentially the same as the pipettes which accompany the Thoma-Zeiss apparatus (see page 209). The capillary portion, however, is graduated, 1, 2/3 and 1/2 which enables the observer to dilute the blood sample in the proportion of 1:200, 1:300 or 1:400 as he may desire. If there is difficulty in drawing in the blood exactly to one of the graduations just mentioned the amount of blood above or below the volume indicated by the graduation may be determined by means of certain delicate cross-lines which are placed directly above and below the graduation. Each cross-line corresponds to 1/100 of the volume of the capillary tube from the tip to the 1 graduation.

A 0.1 per cent solution of sodium carbonate is used to dissolve the stroma of the erythrocytes and so render the blood solution perfectly clear. If this is not done the color of the blood solution invariably appears darker in tone than that of the colored glass wedge. A freshly prepared sodium carbonate solution should be used in order that the clearness of the solution may not be marred by the presence of sodium bicarbonate.

3. **Dare's Hæmoglobinometer** (Fig. 67).—This instrument, as the name signifies, is used for the determination of hæmoglobin. In using either Fleischl's hæmometer or the instrument as modified by Miescher the blood is diluted for examination, whereas with the Dare

instrument *no dilution* is required. This probably allows of rather more accurate determinations than are possible with the old Fleischl apparatus.

The instrument consists essentially of the following parts: (1) A capillary observation cell, (2) a semicircular colored glass wedge, (3) a milled wheel for manipulating the wedge, (4) a candle used to illuminate portions of the capillary observation cell and the colored wedge, (5) a small telescope used in the examination of the areas illuminated by the candle flame, (6) a scale graduated in percentages of the normal amount of hæmoglobin, (7) a hard-rubber case, (8) a movable screen attached to the case.

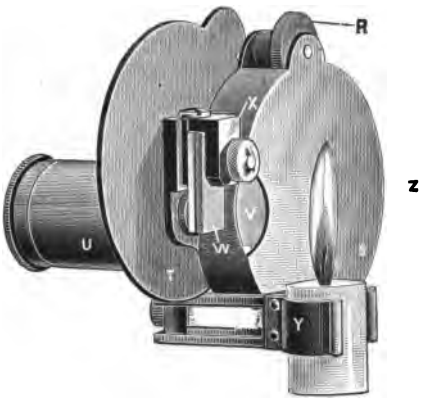


FIG. 67.—DARE'S HÆMOGLOBINOMETER. (*Da Costa.*)

R, Milled wheel acting by a friction bearing on the rim of the color disc; S, case inclosing color disc, and provided with a stage to which the blood chamber is fitted; T, movable wing which is swung outward during the observation, to serve as a screen for the observer's eyes, and which acts as a cover to inclose the color disc when the instrument is not in use; U, telescoping camera tube, in position for examination; V, aperture admitting light for illumination of the color disc; X, capillary blood chamber adjusted to stage of instrument, the slip of opaque glass, W, being nearest to the source of light; Y, detachable candle-holder; Z, rectangular slot through which the hæmoglobin scale indicated on the rim of the color disc is read.

The capillary observation cell is formed of two small, polished rectangular plates of glass, one being transparent and the other opaque. When held in position on the instrument, by means of a small metal bracket, the opaque portion of the cell is nearer the candle and thus serves to soften the glare of light when an observation is being made. The transparent portion of the cell is directly over a circular opening in the case, through which the blood specimen is viewed by means of the small telescope.

The semicircular colored glass wedge is so ground that each particular shade of color corresponds to that possessed by fresh blood which contains some definite percentage of hæmoglobin. It is mounted upon a disc which may be manipulated by the milled wheel in such a manner as to bring successive portions of the wedge in position to be viewed through a circular opening contiguous to the opening through which the blood specimen is viewed. For a further description of the instrument see Figs. 67, 68, and 69.

In using the Dare hæmoglobinometer proceed as follows: Puncture

the finger-tip or lobe of the ear of the subject by means of a needle or scalpel and, after a drop of blood of good proportions has formed, place the flat capillary observation cell in contact with the drop and allow it to fill by capillary attraction (Fig. 69). Replace the cell in its proper place on the instrument. When in position, a portion of this cell may be observed through a small telescope attached to the apparatus. It is viewed through a circular opening and near this circle is a second one through which a portion of a semicircular colored glass wedge is visible. These two circles are illuminated simultaneously by means of the flame of a candle. The colored glass may be rotated by means of a milled wheel and the point of agreement of the color of the adjoining discs may be determined in the same way as in Fleischl's hæmometer. The scale reading gives the percentage of the normal quantity of hæmoglobin which the blood sample under examination contains. Compute the actual hæmoglobin content in the same manner as from the scale reading of the Fleischl hæmometer (see page 204).

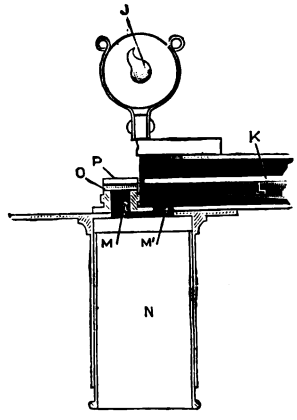


FIG. 68.—HORIZONTAL SECTION OF DARE'S HÆMOGLOBINOMETER. (*Da Costa.*)

4. **Tallquist's Hæmoglobin Scale.**—This consists essentially of a series of ten colors corresponding to stains produced by blood containing varying percentages of hæmoglobin. In using this scale a drop

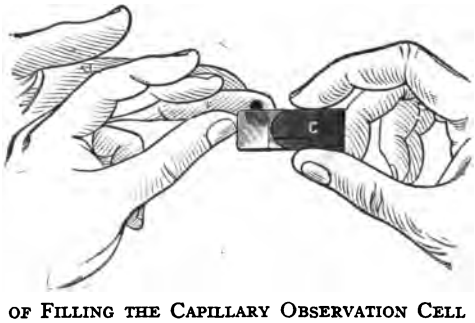


FIG. 69.—METHOD OF FILLING THE CAPILLARY OBSERVATION CELL OF DARE'S HÆMOGLOBINOMETER. (*Da Costa.*)

of blood is allowed to fall on a small section of filter paper and the resulting color is compared with the ten colors of the scale. When the color in the scale is found which corresponds to the color of the blood stain the accompanying hæmoglobin value is read off directly. This

is a very convenient method for determining hæmoglobin at the bedside. There is a possibility of the colors being inaccurately printed, however, and even if originally correct in tint, under the continued influence of air and light they must eventually alter somewhat.

5. **Thoma-Zeiss Hæmocytometer.**—This is an instrument used in "blood counting," *i. e.*, in determining the number of erythrocytes and leucocytes. The instrument consists of a microscopic slide constructed of heavy glass and provided with a central counting cell (see Fig. 70, below). This cell, with the coverglass in position, is exactly 0.1 millimeter deep. The floor of the cell is divided by delicate lines into squares each of which is 1/400 of a square millimeter in area (see Fig. 72, page 210). The volume of blood therefore between any particular square and the coverglass above must be 1/4000 cubic millimeter. Accompanying each instrument are two capillary pipettes

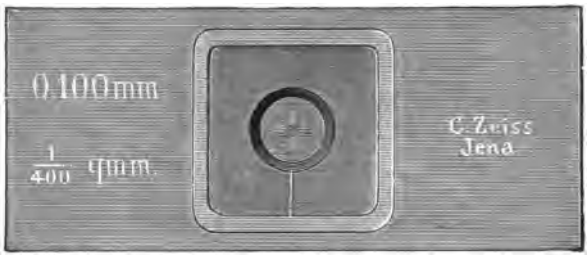


FIG. 70.—THOMA-ZEISS COUNTING CHAMBER. (*Da Costa.*)

(Fig. 71, page 209), each constructed with a mixing bulb in its upper portion. Each bulb is further provided with an enclosed glass bead which is of great assistance in mixing the contents of the chamber. The stem of each pipette is graduated in tenths from the tip to the bulb. The final graduation at the upper end of the bulb is 101 on the pipette used in mixing the blood sample in which the erythrocytes are counted (erythrocytometer, see Fig. 71, page 209), and 11 on the pipette used in mixing the blood sample for the leucocyte count (leucocytometer, see Fig. 71, page 209). In making "blood counts" with the hæmocytometer it is necessary to use some diluting fluid. Two very satisfactory forms of fluid for this purpose are Toison's and Sherrington's solutions.[1] When either of these solutions is used as the diluting fluid

[1] Toison's solution has the following formula:

Methyl-violet	0.025 gram.
Sodium chloride	1 gram.
Sodium sulphate	8 grams.
Glycerol	30 grams.
Distilled water	160 grams.

Sherrington's solution has the following formula:

Methylene-blue	0.1 gram.
Sodium chloride	1.2 gram.
Neutral potassium oxalate	1.2 gram.
Distilled water	300.0 grams.

it is possible to make a very satisfactory count of both the erythrocytes and leucocytes from the same preparation, since the leucocytes are stained by the methyl-violet or methylene-blue.

In counting the erythrocytes by means of the hæmocytometer, proceed as follows: Thoroughly cleanse the tip of the finger or lobe of the ear of the subject by the use of soap and water, alcohol and ether applied in the sequence just given. Puncture the skin by means of a needle or scalpel and allow the blood drop to form without pressure. Place the tip of the pipette in contact with the blood drop, being careful to avoid touching the skin, and draw blood into the pipette up to the point marked 0.5 or 1 according to the desired dilution. Rapidly wipe the tip of the pipette and immediately fill it to the point marked 101 with Toison's or Sherrington's solution. Now thoroughly mix the blood and diluting fluid within the mixing chamber by tapping the pipette gently against the finger, or by shaking it while held securely with the thumb at one end and the middle finger at the other. After the two fluids have been thoroughly mixed the diluting fluid contained in the capillary-tube below the bulb should be discarded in order to insure the collection of a drop of the thoroughly mixed blood and diluting solution for examination. Transfer a drop from the pipette to the ruled floor of the counting chamber and, after placing the coverglass firmly in position,[1] allow an interval of a few minutes to elapse for the corpuscles to settle before making the count. Now place the slide under the microscope and count the number of erythrocytes in a number of squares, counting the corpuscles which are in contact with the upper and the right-hand boundaries of the square as belonging to that square. Take the squares in some definite sequence in order that the recounting of the same corpuscles may be avoided. Of course, all things being equal, the greater the number of squares examined the more accurate the count. It is considered essential under all circumstances, where an accurate count is desired, that the counting chamber shall be filled, at least twice, and

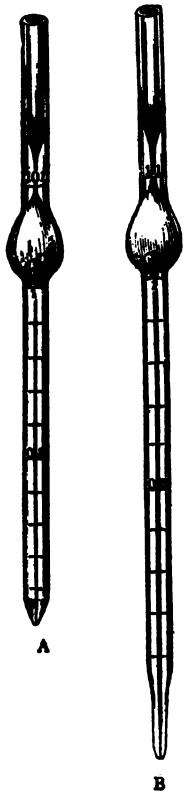


FIG. 71.—THOMA-ZEISS CAPILLARY PIPETTES.
A, Erythrocytometer; B, Leucocytometer.

[1] If the coverglass is in accurate apposition to the counting cell Newton's rings may be plainly observed.

the individual counts made in each instance, as indicated above, before the data are deemed satisfactory.

To calculate the number of erythrocytes per cubic millimeter of undiluted blood proceed as follows: Determine the number of corpuscles in any given number of squares and divide this total by the number of squares, thus obtaining the average number of erythrocytes per square. Multiply this average by 4,000 to obtain the number

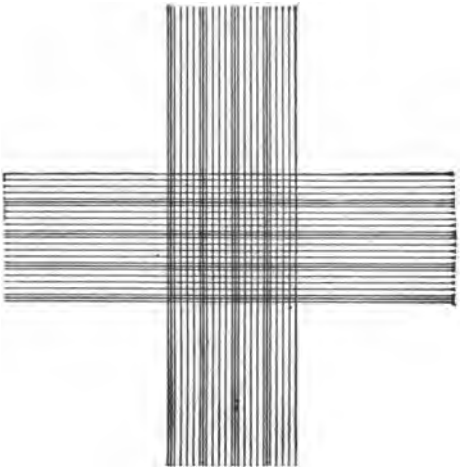


FIG. 72.—ORDINARY RULING OF THOMA-ZEISS COUNTING CHAMBER. (*Da Costa.*)

of erythrocytes per cubic millimeter of *diluted* blood, and multiply this product by 100 or 200, according to the dilution, to obtain the number of erythrocytes per cubic millimeter of *undiluted* blood. Thus:

$$\text{Average number of erythrocytes per square} \times 4{,}000 \times 200 \text{ (or } 100) = \text{Number of erythrocytes per cubic millimeter.}$$

Great care should be taken to see that the capillary pipette is properly cleaned. After using, it should be immediately rinsed out with the diluting fluid, then with water, alcohol, and ether in the sequence given. Finally dry air should be drawn through the capillary and a horse hair inserted to prevent the entrance of dust particles.

In counting leucocytes by means of the hæmocytometer proceed as follows: As mentioned above, if the diluting fluid is either Toison's or Sherrington's solution the leucocytes may be counted in the same specimen of blood in which the erythrocytes are counted. When this is done it is customary to use a slide provided with Zappert's modified ruling (Fig. 73, p. 211). This method is rather more accurate than the older one of counting the leucocytes in a separate specimen

of blood. Furthermore, it is obviously preferable to count both the erythrocytes and the leucocytes from the same blood sample. To insure accuracy the number of leucocytes within the *whole* ruled region

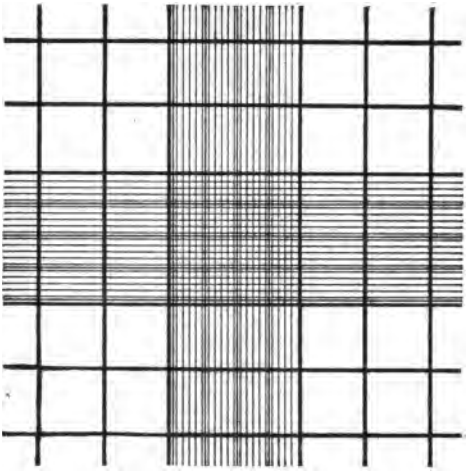


FIG. 73.—ZAPPERT'S MODIFIED RULING OF THOMA-ZEISS COUNTING CHAMBER. (*Da Costa.*)

should be determined in *duplicate* blood samples. This includes the examination of an area eighteen times as great as the old style Thoma-Zeiss central ruling. This region then would correspond to 3,600 of the small squares and, if *duplicate* examinations were made, the total number of small squares examined would aggregate 7,200. The calculation would be as follows:

$$\text{Number of leucocytes in } 7{,}200 \text{ squares} \times 200 \times 4{,}000 \div 7{,}200 = \text{Number of leucocytes per cubic millimeter.}$$

If a Zappert slide is not available, a good plan to follow is to place a diaphragm in the tube of the ocular of the microscope consisting of a circle of black cardboard or metal[1] having a square hole in the center of such a size as to allow of the examination of exactly 100 squares or one-fourth of a square millimeter at one time. With this arrangement any portion of the specimen may be examined and counted whether within or without the ruled area. In counting by means of this device it is, of course, helpful if the microscope is provided with a mechanical stage, but even without this arrangement, if the observer is careful to see that the leucocytes at the extreme boundary of one field move to the opposite boundary when the posi-

[1] Ehrlich's mechanical eye-piece with iris diaphragm is also very satisfactory for this purpose.

tion of the slide is changed, the device may be very satisfactorily employed. The leucocytes should be counted in 36 of the diaphragm-fields in *duplicate* specimens and the calculation made in the same manner as explained above.

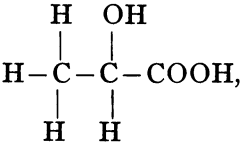
If the leucocytes are counted in a separate specimen of blood ordinarily the diluting fluid is 0.3–0.5 per cent acetic acid, a fluid in which the leucocytes alone remain visible. Under these conditions the dilution is customarily made in the pipette having 11 as the final graduation. The capillary portion is of larger caliber and so requires a greater amount of blood to fill it to the 0.5 or 1 mark than is required in the use of the other form of pipette. In counting the leucocytes according to this method it is customary to draw blood into the pipette up to the 1 mark and immediately fill the remaining portion of the apparatus to the 11 graduation with the 0.3–0.5 per cent acetic acid. It then remains to count the number of leucocytes in the whole central ruled portion of 400 squares. This should be done in *duplicate* samples and the calculation made as follows:

Number of leucocytes in 800 squares. $\times 4{,}000 \times 10 \div 800 =$ Number of leucocytes per cubic millimeter.

CHAPTER XIII.

MILK.

MILK is the most satisfactory individual food material elaborated by nature. It contains the three nutrients, protein, fat, and carbohydrate and inorganic salts in such proportion as to render it a very acceptable dietary constituent. It is a specific product of the secretory activity of the mammary gland. It contains, as the principal solids, *tri-olein*, *tri-palmitin*, *tri-stearin*, *tri-butyrin*, *caseinogen*, *lact-albumin*, *lacto-globulin*, *lactose*, and *calcium phosphate*. It also contains at least traces of lecithin, cholesterol, urea, creatine, creatinine, and the tri-glycerides of caproic, lauric, and myristic acids. Citric acid is also said to be present in milk in minute quantity. Fresh milk is amphoteric in reaction to litmus,[1] but upon standing for a sufficiently long time, unsterilized, it becomes acid in reaction, due to the production of fermentation lactic acid,



from the lactose contained in it. This is brought about through bacterial activity. The white color is imparted to the milk partly through the fine emulsion of the fat and partly through the medium of the caseinogen in solution. The specific gravity of milk varies somewhat, the average being about 1.030. Its freezing-point is about $-0.56°$ C.

Fresh milk does not coagulate on being boiled but a film consisting of a combination of caseinogen forms on the surface. If the film be removed, thus allowing a fresh surface to come in contact with the air, a new film will form indefinitely upon the application of heat. Surface evaporation and the presence of fat facilitate the formation of the film, but are not essential (Rettger). As Jamison and Hertz have shown, a similar film will form on heating any protein

[1] Human milk as well as cow's milk. It is, however, acid to phenolphthalein.

solution containing fat or paraffin. If the milk is acid in reaction, through the inception of lactic acid fermentation, or from any other cause, no film will form when heat is applied, but instead a true coagulation will occur. When milk is boiled certain changes occur in its odor and taste. These changes, according to Rettger, are due to a partial decomposition of the milk proteins and are accompanied by the liberation of a volatile sulphide, probably hydrogen sulphide.

The milk-curdling enzymes of the gastric and the pancreatic juice have the power of splitting the caseinogen of the milk, through a process of hydrolysis, into *soluble casein* and a *peptone-like* body.

FIG. 74.—NORMAL MILK AND COLOSTRUM.
a, Normal milk; *b*, Colostrum.

This soluble casein then forms a combination with the calcium of the milk and an insoluble curd of *calcium casein* or *casein* results. The clear fluid surrounding the curd is known as *whey*.

The most pronounced difference between human milk and cow's milk is in the protein content, although there are also differences in the fats and likewise striking biological differences difficult to define chemically. It has been shown that the caseinogen of human milk differs from the caseinogen of cow's milk in being more difficult to precipitate by acid or coagulate by gastric rennin. The casein curd also forms in a much looser and more flocculent manner than that from cow's milk and is for this reason much more easily digested than the latter. Interesting data relative to the composition of milk from various sources may be gathered from the following table which was compiled mainly from the results of investigations by Bunge and by Abderhalden. It will be noted that the composition of the milk

varies directly with the length of time needed for the young of the particular species to double in weight.

Species.	Period in which Weight of the New-born is Doubled (Days).	100 Parts of Milk Contain			
		Proteins.	Salts.	Calcium.	Phosphoric Acid.
Man	180	1.6	0.2	0.033	0.047
Horse	60	2.0	0.4	0.124	0.131
Cow	47	3.5	0.7	0.160	0.197
Goat	22	3.7	0.8	0.197	0.284
Sheep	15	4.9	0.8	0.245	0.293
Pig	14	5.2	0.8	0.249	0.308
Cat	9.5	7.0	1.0	.....	.....
Dog	9	7.4	1.3	0.455	0.508
Rabbit	6	10.4	2.5	0.891	0.997

Lactose, the principal carbohydrate constituent of milk, is an important member of the disaccharide group. It occurs only in milk, except as it is found in the urine of women during pregnancy,

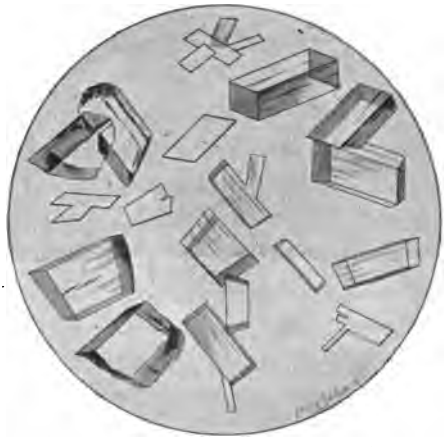


FIG. 75.—LACTOSE.

during the nursing period, and soon after weaning; it also occurs in the urine of normal persons after the ingestion of a very large amount of lactose in the food. It is not derived directly from the blood, but is a specific product of the cellular activity of the mammary gland. It has strong reducing power, is dextro-rotatory, and forms an osazone with phenylhydrazine. The souring of milk is due to the formation of lactic acid from lactose through the agency of the *bacterium lactis*.

Putrefactive bacteria in the alimentary canal may bring about this same reaction. Lactose is *not* fermentable by pure yeast. It was recently claimed that *lactosin*, a new carbohydrate, had been isolated from milk.

Caseinogen, the principal protein constituent of milk, belongs to the group of phosphoproteins. It has acidic properties and combines with bases to produce salts. It is not coagulable upon boiling and is precipitated from its neutral solution by certain metallic salts as well as upon saturation with sodium chloride or magnesium sulphate. Its acid solution is precipitated by an excess of mineral acid.

Lactalbumin and lacto-globulin, the protein constituents of milk, next in importance to caseinogen, closely resemble serum albumin and serum globulin in their general properties. According to Wroblewski, a protein called *opalisin* is also present in milk.

Colostrum is the name given to the product of the mammary gland secreted for a short time before parturition and during the early period of lactation (see Fig. 74, p. 214). It is yellowish in color, contains more solid matter than ordinary milk, and has a higher specific gravity (1.040–1.080). The most striking difference between colostrum and ordinary milk is the high percentage of lactalbumin and lacto-globulin in the former. This abnormality in the protein content is responsible for the coagulation of colostrum upon boiling.

Such enzymes as lipase, amylase, galactase, catalase, oxidases, peroxidases, and reductases have been identified in milk, but not all of them in milk of the same species of animal.

Among the principal preservatives used in connection with milk are formaldehyde, hydrogen peroxide, boric acid, borates, salicylic acid, and salicylates.

Experiments on Milk.

1. **Reaction.**—Test the reaction of fresh cow's milk to litmus.

2. **Biuret Test.**—Make the biuret test according to directions given on page 90.

3. **Microscopical Examination.**—Examine fresh *whole* milk, *skimmed or centrifugated* milk, and *colostrum* under the microscope. Compare the microscopical appearance with Fig. 74, page 214.

4. **Specific Gravity.**—Determine the specific gravity of both whole and skimmed milk (see p. 254). Which possesses the higher specific gravity? Explain why this is so.

5. **Film Formation.**—Place 10 c.c. of milk in a small beaker

and boil a few minutes. Note the formation of a film. Remove the film and heat again. Does the film now form? Of what substance is this film composed? The biuret test was positive, why do we not get a coagulation here when we heat to boiling?

6. **Coagulation Test.**—Place about 5 c.c. of milk in a test-tube, acidify slightly with dilute acetic acid and heat to boiling. Do you get any coagulation? Why?

7. **Action of Hot Alkali.**—To a little milk in a test-tube add a few drops of potassium hydroxide and heat. A yellow color develops and gradually deepens into a brown. To what is the formation of this color due?

8. **Test for Chlorides.**—To about 5 c.c. of milk in a test-tube add a few drops of *very dilute* nitric acid to form a precipitate. Filter off this precipitate and test the filtrate for chlorides. Does milk contain any chlorides?

9. **Guaiac Test.**—To about 5 c.c. of water in a test-tube add 3 drops of milk and enough alcoholic solution of guaiac (strength about 1:60)[1] to cause a turbidity. Thoroughly mix the fluids by shaking and observe any change which may gradually take place in the color of the mixture. If no blue color appears in a short time, heat the tube gently below 60° C. and observe whether the color reaction is hastened. In case a blue color does not appear in the course of a few minutes, add hydrogen peroxide or old turpentine, drop by drop, until the color is observed. Fresh milk will frequently give this blue color when treated with an alcoholic solution of guaiac without the addition of hydrogen peroxide or old turpentine. See discussion on page 188.

10. **Tests to Differentiate Between Raw Milk and Heated Milk.**—(*a*) *Kastle's Peroxidase Reaction.*—The peroxidase reaction of milk is founded upon the fact that small amounts of *raw* milk will induce the oxidation of various leuco compounds by hydrogen peroxide. This reaction has been used in a practical way as the most convenient means of differentiating between *raw milk* and *heated milk.* Many substances have been employed for this purpose, *e. g.*, guaiac, paraphenylenediamine, ortol, amidol, etc. Kastle has found that a dilute solution of "trikresol"[2] acts as a sensitizing agent in the peroxidase reaction and offers the following test which is based upon this fact: To 2–5 c.c. of *raw milk* in a test-tube add 0.1–0.3 c.c. of M/10

[1] Buckmaster advises the use of an alcoholic solution of guaiaconic acid instead of an alcoholic solution of guaiac resin. Guaiaconic acid is a constituent of guaiac resin.

[2] "Trikresol" is the trade name of an antiseptic which contains the three cresols in approximately equal proportions.

hydrogen peroxide and 1 c.c. of a 1 per cent solution of "trikresol." A slight though unmistakable *yellow color* will be observed to develop throughout the solution.

Repeat the test using milk which has been boiled or heated to 80° C. for 10–20 minutes, and cooled, and note that no yellow color is produced.

The color reaction in the case of the raw milk probably results from the oxidation of the cresols by the hydrogen peroxide. The first product of this oxidation[1] then oxidizes the leuco compound, when such is present, and causes the color observed.

(*b*) *Wilkinson and Peters' Test.*[2]—To 10 c.c. of the milk to be tested add 2 c.c. of a 4 per cent alcoholic solution of benzidine, sufficient acetic acid to coagulate the milk (usually 2–3 drops) and finally 2 c.c. of a 3 per cent solution of hydrogen peroxide. Raw milk yields an immediate blue color. In adding the peroxide it is best to permit it to flow slowly down the wall of the vessel containing the mixture instead of allowing it to mix with the milk. Milk which has been heated to 78° C. or above remains unchanged.

11. **Saturation with Magnesium Sulphate.**—Place about 5 c.c. of milk in a test-tube and saturate with solid magnesium sulphate. What is this precipitate?

12. **Influence of Gastric Rennin on Milk.**—Prepare a series of five tubes as follows:

(*a*) 5 c.c. of fresh milk + 0.2 per cent HCl (add drop by drop until a precipitate forms).

(*b*) 5 c.c. of fresh milk + 5 drops of *rennin* solution.

(*c*) 5 c.c. of fresh milk + 10 drops of 0.5 per cent Na_2CO_3.

(*d*) 5 c.c. of fresh milk + 10 drops of ammonium oxalate.

(*e*) 5 c.c. of fresh milk + 5 drops of 0.2 per cent HCl.

Now to each of the tubes (*c*), (*d*) and (*e*) add 5 drops of *rennin* solution. Place the whole series of five tubes at 40° C. and after 10–15 minutes note what is occurring in the different tubes. Give a reason for each particular result.

13. **Preparation of Caseinogen.**—Fill a large beaker one-third full of *skimmed* (or centrifugated) milk and dilute it with an equal volume of water. Add dilute hydrochloric acid until a flocculent precipitate forms. Stir after each acidification and do not add an excess of the acid as the precipitate would dissolve. Allow the precipitate to settle, decant the supernatant fluid, and reserve it for use in

[1] Probably some organic peroxide or quinoid compound.

[2] Wilkinson and Peters: *Z. Nahr-Genussm.*, XVI, No. 3, p. 172.

later (14–16) experiments. Filter off the precipitate of caseinogen and remove the excess of moisture by pressing it between filter papers. Transfer the caseinogen to a small beaker, add enough 95 per cent alcohol to cover it and stir for a few moments. Filter, and press the precipitate between filter papers to remove the alcohol. Transfer the caseinogen again to a small *dry* beaker, cover the precipitate with ether and heat on a water-bath for ten minutes, stirring continuously. Filter (reserve the filtrate), and press the precipitate as dry as possible between filter papers. Open the papers and allow the ether to evaporate spontaneously. Grind the precipitate to a powder in a mortar. Upon the caseinogen prepared in this way make the following tests:

(*a*) *Solubility.*—Try the solubility in the ordinary solvents.

(*b*) *Millon's Reaction.*—Make the test according to the directions given on page 88.

(*c*) *Biuret Test.*—Make the test according to directions given on page 90.

(*d*) *Hopkins-Cole Reaction.*—Make the test according to the directions given on page 89.

(*e*) *Loosely Combined Sulphur.*—Test for loosely combined sulphur according to the directions given on page 100.

(*f*) *Fusion Test for Phosphorus.*—Test for phosphorus by fusion according to directions given on page 247.

14. **Coagulable Proteins of Milk.**—Place the filtrate from the original caseinogen precipitate in a casserole and heat, on a wire gauze, over a free flame. As the solution concentrates, a coagulum consisting of *lactalbumin* and *lactoglobulin* will form. Continue to concentrate the solution until the volume is about one-half that of the original solution. Filter off the coagulable proteins (reserve the filtrate) and test them as follows:

(*a*) *Millon's Reaction.*—Make the test according to the directions given on page 88.

(*b*) *Biuret Test.*—Make the test according to the directions given on page 90.

(*c*) *Hopkins-Cole Reaction.*—Make the test according to the directions given on page 89.

15. **Detection of Calcium Phosphate.**—Evaporate the filtrate from the coagulable proteins, on a water-bath, until crystals begin to form. It may be necessary to concentrate to 15 c.c. before any crystallization will be observed. Cool the solution, filter off the crystals (reserve the filtrate), and test them as follows:

(*a*) *Microscopical Examination.*—Examine the crystals and compare them with those in Fig. 76.

(*b*) Dissolve the crystals in nitric acid. Test part of the acid solution for phosphates. Render the remainder of the solution slightly alkaline with ammonia, then acidify with acetic acid and add ammonium oxalate. Examine the crystals under the microscope and compare them with those in Fig. 99, p. 340.

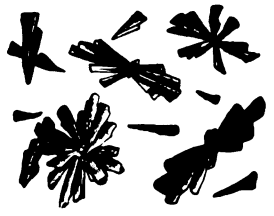


FIG. 76.—CALCIUM PHOSPHATE.

16. **Detection of Lactose.**—Concentrate the filtrate from the calcium phosphate until it is of a syrup-like consistency. Allow it to stand over night and observe the formation of crystals of lactose. Make the following experiments.

(*a*) *Microscopical Examination.*—Examine the crystals and compare them with those in Fig. 75, page 215.

(*b*) *Fehling's Test.*—Try Fehling's test upon the mother liquor.

(*c*) *Phenylhydrazine Test.*—Apply the phenylhydrazine test to some of the mother liquor according to the directions given on page 23.

17. **Milk Fat.**—(*a*) Evaporate the ether filtrate from the caseinogen (Experiment 13) and observe the fatty residue. The milk fat was carried down with the precipitate of caseinogen and was removed when the latter was treated with ether. If centrifugated milk was used in the preparation of the caseinogen the amount of fat in the ether filtrate may be very small. To secure a larger yield of fat proceed according to directions given under (*b*) below.

(*b*) To 25 c.c. of whole milk in an evaporating dish add a little sand or filter paper and evaporate the fluid to dryness on a water-bath. Grind or break up the residue after cooling and extract with ether in a flask. Filter and remove the ether from the filtrate by evaporation. How can you identify fats in the ethereal residue?

18. **Saponification of Butter.**—Dissolve a small amount of butter in alcohol made strongly alkaline with potassium hydroxide. Place the alcoholic-potash solution in a casserole, add about 100 c.c. of water and boil for 10–15 minutes or until the odor of alcohol cannot be detected. Place the casserole in a hood and neutralize the solution with sulphuric acid. Note the odor of volatile fatty acids, particularly butyric acid.

19. **Detection of Preservatives.**—(*a*) *Formaldehyde.*

I. *Gallic Acid Test.*—Acidify 30 c.c. of milk with 2 c.c. of normal sulphuric acid and distil. Add 0.2–0.3 c.c. of a saturated alcoholic

solution of gallic acid to the *first* 5 c.c. of the distillate, then incline the test-tube and slowly introduce 3–5 c.c. of concentrated sulphuric acid, allowing it to run slowly down the side of the tube. A green ring, which finally changes to blue, is formed at the juncture of the fluids. This is claimed, by Sherman, to be twice as delicate as either the sulphuric acid or the hydrochloric acid test for formaldehyde.

II. *Leach's Hydrochloric Acid Test.*—Mix 10 c.c. of milk and 10 c.c. of concentrated hydrochloric acid containing about 0.002 gram of ferric chloride in a small porcelain evaporating dish or casserole and *gradually* raise the temperature of the mixture, on a water-bath, nearly to the boiling-point, with occasional stirring. If formaldehyde is present a violet color is produced, while a brown color develops in the absence of formaldehyde. In case of doubt the mixture, after having been heated nearly to the boiling-point for about one minute, should be diluted with 50–75 c.c. of water, and the color of the diluted fluid carefully noted, since the violet color if present will quickly disappear. Formaldehyde may be detected by this test when present in the proportion 1 :250,000.

(*b*) *Salicylic and Salicylates.*—Remont's Method.[1] Acidify 20 c.c. of milk with sulphuric acid, shake well to break up the curd, add 25 c.c. of ether, mix thoroughly, and allow the mixture to stand. By means of a pipette remove 5 c.c. of the ethereal extract, evaporate it to dryness, boil the residue with 10 c.c. of 40 per cent alcohol, and cool the alcoholic solution. Make the volume 10 c.c., filter through a *dry* paper if necessary to remove fat, and to 5 c.c. of the filtrate, which represents 2 c.c. of milk, add 2 c.c. of a 2 per cent solution of ferric chloride. The production of a *purple* or *violet* color indicates the presence of salicylic acid.

This test may form the basis of a quantitative method by diluting the final solution to 50 c.c. and comparing this with standard solutions of salicylic acid. The colorimetric comparisons may be made in a Duboscq colorimeter.

(*c*) *Hydrogen Peroxide.*—Add 2–3 drops of a 2 per cent aqueous solution of para-phenylenediamine hydrochloride to 10–15 c.c. of milk. If hydrogen peroxide is present a *blue* color will be produced immediately upon shaking the mixture or after allowing it to stand for a few minutes. It is claimed that hydrogen peroxide may be detected by this test when present in the proportion 1 : 40,000.

(*d*) *Boric Acid and Borates.*—To the ash, obtained according to the directions given in Experiment 4, page 407, add 2 drops of

[1] Sherman's Organic Analysis, p. 232.

dilute hydrochloric acid and 1 c.c. of water. Place a strip of turmeric paper in the dish and after allowing it to soak for about one minute remove it and allow it to dry in the air. The presence of boric acid is indicated by the production of a deep *red* color which changes to *green* or *blue* upon treatment with a dilute alkali. This test is supposed to show boric acid when present in the proportion 1 : 8000.

CHAPTER XIV.

EPITHELIAL AND CONNECTIVE TISSUES.

EPITHELIAL TISSUE (KERATIN).

THE albuminoid *keratin* constitutes the major portion of hair, horn, hoof, feathers, nails, and the epidermal layer of the skin. There is a group of keratins the members of which possess very similar properties. The keratins as a group are insoluble in the usual protein solvents and are not acted upon by the gastric or pancreatic juices. They all respond to the xanthoproteic and Millon reactions and are characterized by containing large amounts of sulphur. Keratin from any of its sources may be prepared in a pure form by treatment, in sequence, with artificial gastric juice, artificial pancreatic juice, boiling alcohol, and boiling ether, from twenty-four to forty-eight hours being devoted to each process.

EXPERIMENTS ON EPITHELIAL TISSUE.

Keratin.

Horn shavings or nail parings may be used in the experiments which follow:

1. *Solubility.*—Test the solubility of keratin in the ordinary solvents (see p. 22).
2. *Millon's Reaction.*
3. *Xanthoproteic Reaction.*
4. *Adamkiewicz's Reaction.*
5. *Hopkins-Cole Reaction.*
6. *Test for Loosely Combined Sulphur.*

CONNECTIVE TISSUE.

I. WHITE FIBROUS TISSUE.

The principal solid constituent of white fibrous connective tissue is the albuminoid collagen. This body is also found in smaller percentage in cartilage, bone, and ligament, but the collagen from the various sources is not identical in composition. In common with the

keratins, collagen is insoluble in the usual protein solvents. It differs from keratin in containing less sulphur. One of the chief characteristics of collagen is, according to Hofmeister, the property of being hydrolyzed by boiling acid or water with the formation of *gelatin*. Emmett and Gies claim that under these conditions there is an intramolecular rearrangement of collagen and the resultant gelatin is consequently not the product of hydrolysis. The liberation of ammonia from the collagen during the process apparently confirms this view. Collagen gives Millon's reaction as well as the xanthoproteic and biuret tests.

The form of white fibrous tissue most satisfactory for general experiments is the *tendo Achillis* of the ox. According to Buerger and Gies the fresh tissue has the following composition:

Water	62.87%
Solids	37.13
Inorganic matter	0.47
Organic matter	36.66
Fatty substance (ether-soluble)	1.04
Coagulable protein	0.22
Mucoid	1.28
Elastin	1.63
Collagen	31.59
Extractives, etc.	0.90

The mucoid mentioned above is called *tendomucoid* and is a glycoprotein. It possesses properties similar to those of other connective tissue mucoids, *e. g.*, osseomucoid and chondromucoid.

Gelatin, the body which results from the hydrolysis of collagen (see statement of Emmett and Gies above), is also an albuminoid. It responds to nearly all the protein tests. It differs from the keratins and collagen in being easily digested and absorbed. Gelatin is not a satisfactory substitute for the protein constituents of a normal diet, however, since a certain portion of its nitrogen is not available for the uses of the organism. Gelatin from cartilage differs from the gelatin from other sources in containing a lower percentage of nitrogen. Tyrosine and tryptophane are not numbered among the decomposition products of gelatin, hence it does not respond to Millon's reaction or the Hopkins-Cole reaction.

Experiments on White Fibrous Tissue.

The *tendo Achillis* of the ox may be taken as a satisfactory type of the white fibrous connective tissue.

1. **Preparation of Tendomucoid.**—Dissect away the fascia from about the tendon and cut the clean tendon into small pieces. Wash the

pieces in water, changing the wash water often in order to remove as much as possible of the soluble protein and inorganic salts. Transfer the washed pieces of tendon to a flask and add 300 c.c. of *half-saturated* lime water.[1] Shake the flask at intervals for twenty-four hours. Filter off the pieces of tendon and precipitate the mucoid with dilute hydrochloric acid. Allow the mucoid precipitate to settle, decant the supernatant fluid and filter the remainder. Test the mucoid as follows:

(*a*) *Solubility.*—Try the solubility in the ordinary solvents (see page 22).

(*b*) *Biuret Test.*—First dissolve the mucoid in potassium hydroxide solution and then add a dilute solution of cupric sulphate.

(*c*) *Test for Loosely Combined Sulphur.*

(*d*) *Hydrolysis of Tendomucoid.*—Place the remainder of the mucoid in a small beaker, add about 30 c.c. of water and 2 c.c. of dilute hydrochloric acid and boil until the solution becomes dark brown. Cool the solution, neutralize it with *solid* potassium hydroxide, and test by Fehling's test. With a reduction of Fehling's solution and a positive biuret test what do you conclude regarding the nature of tendomucoid?

2. **Collagen.**—This substance is present in the tendon to the extent of about 32 per cent. Therefore in making the following tests upon the pieces of tendon from which the mucoid, soluble protein, and inorganic salts were removed in the last experiment, we may consider the tests as being made upon *collagen.*

(*a*) *Solubility.*—Cut the collagen into very fine pieces and try its solubility in the ordinary solvents (see page 22).

(*b*) *Millon's Reaction.*

(*c*) *Biuret Test.*

(*d*) *Xanthoproteic Reaction.*

(*e*) *Hopkins-Cole Reaction.*

(*f*) *Test for Loosely Combined Sulphur.*—Take a *large* piece of collagen in a test-tube and add about 5 c.c. of potassium hydroxide solution. Heat until the collagen is partly decomposed, then add 1–2 drops of plumbic acetate and again heat to boiling.

(*g*) *Formation of Gelatin from Collagen.*—Transfer the remainder of the pieces of collagen to a casserole, fill the vessel about two-thirds full of water and boil for several hours, adding water at intervals as needed. By this means the collagen is transformed and a body known as *gelatin* is produced (see p. 224).

[1] Made by mixing equal volumes of *saturated* lime water and water from the faucet.

3. **Gelatin.**—On the gelatin formed from the transformation of collagen in the above experiment (*g*), or on gelatin furnished by the instructor make the following tests:

(*a*) *Solubility.*—Try the solubility in the ordinary solvents (see page 22) and in *hot* water.

(*b*) *Millon's Reaction.*

(*c*) *Hopkins-Cole Reaction.*—Conduct this test according to the modification given on page 99.

(*d*) *Test for Loosely Combined Sulphur.*

Make the following tests upon a *solution* of gelatin in hot water:

(*a*) *Precipitation by Mineral Acids.*—Is it precipitated by strong mineral acids such as concentrated hydrochloric acid?

(*b*) *Salting-out Experiment.*—Saturate a little of the solution with solid ammonium sulphate. Is the gelatin precipitated? Repeat the experiment with sodium chloride. What is the result?

(*c*) *Precipitation by Metallic Salts.*—Is it precipitated by metallic salts such as cupric sulphate, mercuric, and plumbic acetate?

(*d*) *Coagulation Test.*—Does it coagulate upon boiling?

(*e*) *Precipitation by Alkaloidal Reagents.*—Is it precipitated by such reagents as picric acid, tannic acid, and trichloracetic acid?

(*f*) *Biuret Test.*—Does it respond to the biuret test?

(*g*) *Bardach's Reaction.*—Does it yield the typical crystals of this reaction? (See page 92.)

(*h*) *Precipitation by Alcohol.*—Fill a test-tube one-half full of 95 per cent alcohol and pour in a small amount of *concentrated* gelatin solution. Do you get a precipitate? How would you prepare pure gelatin from the *tendo Achillis* of the ox?

II. YELLOW ELASTIC TISSUE (ELASTIN).

The *ligamentum nuchæ* of the ox may be taken as a satisfactory type of the yellow elastic connective tissue. The principal solid constituent of this tissue is *elastin*, a member of the albuminoid group. In common with the keratins and collagen, elastin is an insoluble body and gives the protein color reactions. It differs from keratin principally in the fact that it may be digested by enzymes and that it contains a very small amount of sulphur.

Yellow elastic tissue also contains mucoid and collagen but these are present in much smaller amount than in white fibrous tissue, as may be seen from the following percentage composition of the fresh *ligamentum nuchæ* of the ox as determined by Vandegrift and Gies:

Water		57.57%
Solids		42.43
Inorganic matter	0.47	
Organic matter	41.96	
Fatty substance (ether-soluble)	1.12	
Coagulable protein	0.62	
Mucoid	0.53	
Elastin	31.67	
Collagen	7.23	
Extractives, etc.	0.80	

Experiments on Elastin.

1. **Preparation of Elastin (Richards and Gies).**—Cut the ligament into fine strips, run it through a meat chopper and wash the finely divided material in cold, running water for 24–48 hours. Add an excess of *half-saturated* lime water (see note at the bottom of p. 225) and allow the hashed ligament to extract for 48–72 hours. Decant the lime water, remove all traces of alkali by washing in water and then boil in water with repeated renewals until only traces of protein material can be detected in the wash water. Decant the fluid and boil the ligament in 10 per cent acetic acid for a few hours. Treat the pieces with 5 per cent hydrochloric acid at room temperature for a similar period, extract again in *hot* acetic acid and in *cold* hydrochloric acid. Wash out traces of acid by means of water and then thoroughly dehydrolyze by boiling alcohol and boiling ether in turn. Dry in an air-bath and grind to a powder in a mortar.

2. **Solubility.**—Try the solubility of the finely divided elastin, prepared by yourself or furnished by the instructor, in the ordinary solvents (see page 22). How does its solubility compare with that of collagen?

3. **Millon's Reaction.**

4. **Xanthoproteic Reaction.**

5. **Biuret Test.**

6. **Hopkins-Cole Reaction.**—Conduct this test according to the modification given on page 99.

7. **Test for Loosely Combined Sulphur.**

III. CARTILAGE.

The principal solid constituents of the matrix of cartilaginous tissue are *chondromucoid, chondroitin-sulphuric acid, chondroalbumoid* and *collagen*. Chondromucoid differs from the mucoids isolated from

other connective tissues in the large amount of chondroitin-sulphuric acid obtained upon decomposition. Besides being an important constituent of all forms of cartilage, chondroitin-sulphuric acid has been found in bone, ligament, the mucosa of the pig's stomach, the kidney of the ox, the inner coats of large arteries and in human urine. It may be decomposed through the action of acid and yields a nitrogenous body known as *chondroitin* and later this body yields *chondrosin.* Chondrosin is also a nitrogenous body and has the power of reducing Fehling's solution more strongly than dextrose. Sulphuric acid is a by-product in the formation of chondroitin, and acetic acid is a by-product in the formation of chondrosin.

Chondroalbumoid is similar in some respects to elastin and keratin. It differs from keratin in being soluble in gastric juice and in containing considerably less sulphur than any member of the keratin group. It gives the usual protein color reactions.

Experiments on Cartilage.

1. **Preparation of the Cartilage.**—Boil the trachea of an ox in water until the cartilage rings may be completely freed from the surrounding tissue. Use the cartilage so obtained in the following experiments:

2. **Solubility.**—Cut one of the rings into very small pieces and try the solubility of the cartilage in the ordinary solvents (see page 22).

3. **Millon's Reaction.**

4. **Xanthoproteic Reaction.**

5. **Hopkins-Cole Reaction.**—Conduct this test according to the modification given on page 99.

6. **Test for Loosely Combined Sulphur.**

7. **Preparation of Cartilage Gelatin.**—Cut the remaining cartilage rings into small pieces, place them in a casserole with water and boil for several hours. Filter while the solution is still hot. Observe that the filtrate soon becomes more or less solid. What is the reason for this? Bring a portion of the material into solution by heat and try the following tests:

(*a*) *Biuret Test.*

(*b*) *Bardach's Reaction.*

(*c*) *Test for Loosely Combined Sulphur.*

(*d*) To about 5 c.c. of the solution in a test-tube add a few drops of barium chloride. Do you get a precipitate, and if so to what is the precipitate due?

(*e*) To about 5 c.c. of the solution in a test-tube add a few drops of dilute hydrochloric acid and boil for a few moments. Now add a little barium chloride to this solution. Is the precipitate any larger than that obtained in the preceding experiment? Why?

(*f*) To the remainder of the solution add a little dilute hydrochloric acid and boil for a few moments. Cool the solution, neutralize with *solid* potassium hydroxide, and try Fehling's test. Explain the result.

IV. OSSEOUS TISSUE.

Bone is composed of about equal parts of organic and inorganic matter. The organic portion, called *ossein*, may be obtained by removing the inorganic salts through the medium of dilute acid. Ossein is practically the same body which is termed collagen in the other connective tissues, and in common with collagen yields gelatin upon being boiled with dilute mineral acid.

In common with the other connective tissues bone contains a mucoid and an albumoid. Because of their origin these bodies are called *osseomucoid* and *osseoalbumoid*. Osseomucoid, when boiled with hydrochloric acid, yields sulphuric acid and a substance capable of reducing Fehling's solution. The composition of osseomucoid is very similar to that of tendomucoid and chondromucoid (see page 104).

Experiment on Osseous Tissue.

Analysis of Bone Ash.—Take 1 gram of bone ash in a small beaker and add a little dilute nitric acid. What does the effervescence indicate? Stir thoroughly and when the major portion of the ash is dissolved add an equal volume of water and filter. To the acid filtrate add ammonium hydroxide to alkaline reaction. A heavy white precipitate of phosphates results. (What phosphates are precipitated here by the ammonia?) Filter and test the filtrate for chlorides, sulphates, phosphates, and calcium. Add dilute acetic acid to the precipitate on the paper and test this filtrate for calcium and phosphates. To the precipitate remaining undissolved on the paper add a little dilute hydrochloric acid and test this last filtrate for phosphates and iron.

Reference to the following scheme may facilitate the analysis.

BONE ASH.

Add dilute nitric acid, stir thoroughly and after the major portion of the ash has been brought into solution add a little distilled water and filter.

Residue I.
(discard)

Filtrate I.
Add ammonium hydroxide to alkaline reaction and filter.

Residue II.
Treat *on paper* with acetic acid.

Filtrate II.
Test for:
1. Chlorides.
2. Sulphates.
3. Phosphates.
4. Calcium.

Residue III.
Treat *on paper* with hydrochloric acid.

Filtrate III.
Test for:
1. Phosphates.
2. Calcium.

Filtrate IV.
Test for:
1. Iron.
2. Phosphates.

V. ADIPOSE TISSUE.

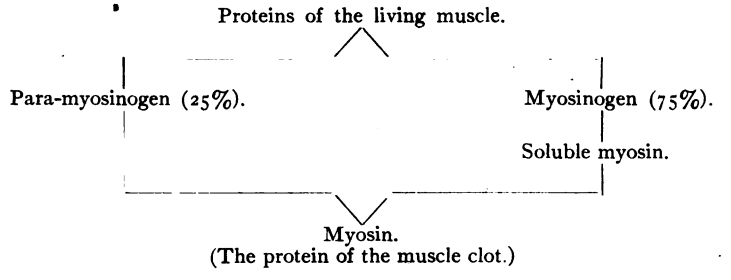
For discussion and experiments see the chapter on Fats, page 128.

CHAPTER XV.

MUSCULAR TISSUE.

THE muscular tissues are divided physiologically into the voluntary (striated) and the involuntary (non-striated). In the chemical examination of muscular tissue the voluntary form is generally employed. Muscle contains about 25 per cent of solid matter, of which about four-fifths is protein material and the remaining one-fifth extractives and inorganic salts.

The proteins are the most important of the constituents of muscular tissue. In the living muscle we find two proteins, *myosinogen* and *para-myosinogen*. These may be shown to be present in *muscle plasma* expressed from fresh muscles. In common with the plasma of the blood this muscle plasma has the power of coagulating, and the clot formed in this process is called *myosin*. In the onset of *rigor mortis* we have an indication of the formation of this myosin clot within the body. The relation between the proteins of *living* and *dead* muscle is represented graphically by Halliburton as follows:



Of the total protein content of living muscle about 75 per cent is made up by the *myosinogen* and the remaining 25 per cent is para-myosinogen. These proteins may be separated by subjecting the muscle plasma to fractional coagulation in the usual way. Under these conditions the para-myosinogen is found to coagulate at 47° C. and the myosinogen to coagulate at 56° C. It is also claimed by some investigators that it is possible to separate these two proteins by the fractional ammonium sulphate method, but the possibility of making

an accurate separation by this method is somewhat doubtful. It is well established that para-myosinogen is a globulin since it responds to certain of the protein precipitation tests and is insoluble in water. Myosinogen, on the contrary, is not a typical globulin since it is soluble in water. It has been called a *pseudo-globulin*. Myosin possesses the globulin characteristics. It is insoluble in water but soluble in the other protein solvents and is precipitated from its solution upon saturation with sodium chloride.

Very recently Mellanby has reported observations which he claims indicate that there is only *one* protein in muscle and that *rigor mortis* is due to the coagulation of this protein under the combined influences of the salt present in the muscle and the lactic acid developed upon the death of the muscle. He further states that the disappearance of rigor is due to the fact that the lactic acid which is continually formed brings this protein into solution.

Under the name *extractives* we class a number of muscle constituents which occur in traces in the tissue and may be extracted by water, alcohol, or ether. There are two classes of these extractives, the *non-nitrogenous extractives* and the *nitrogenous extractives*. Grouped under the non-nitrogenous bodies we have *glycogen*, *dextrin*, *sugars*, *lactic acid*, *inosite*, $C_6H_6(OH)_6$, and fat. In the class of nitrogenous extractives we have *creatine*, *creatinine*, *xanthine*, *hypoxanthine*, *uric acid*, *urea*, *carnine*, *guanine*, *phosphocarnic acid*, *inosinic acid*, *carnosine*, *taurine*, *carnitine*, *novaine*, *ignotine*, *neosine*, *oblitine*, *carnomuscarine* and *methylguanidine* (see formulas on page 236). Not all of these extractives are present in the muscles of all species of animals. Other extractives besides those enumerated above have been described and there are undoubtedly still others whose presence remains undetermined. A detailed consideration would, however, be unprofitable in this place.

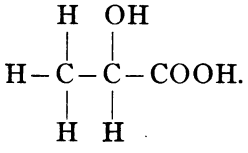
Glycogen is an important constituent of muscle. The content of this polysaccharide in muscle varies and is markedly decreased by intense muscular activity. It is transformed into sugar and used as fuel. The liver is the organ which stores the reserve supply of glycogen and transforms it into dextrose which is passed into the blood stream and so carried to the working muscle where it is synthesized into glycogen. The glycogen thus formed is then changed into dextrose as the working muscle may need it.

Glycogen is a polysaccharide and has the same percentage composition as starch and dextrin. It resembles starch in forming an opalescent solution and resembles dextrin in being very soluble, in

giving a reddish color with iodine and in being dextro-rotatory. Glycogen may be prepared from muscle by extracting with boiling water and then precipitating the glycogen from the aqueous solution by alcohol: dilute or concentrated potassium hydroxide may also be used to extract the glycogen. Glycogen may be prepared in the form of a white, tasteless, amorphous powder. It is completely precipitated from its solution by saturation with solid ammonium sulphate, but is not precipitated by saturation with sodium chloride. It may also be precipitated by alcohol, tannic acid, or ammoniacal basic lead acetate. It has the power of holding cupric hydroxide in solution in alkaline fluids but cannot reduce it. It may be hydrolyzed with the formation of dextrose by dilute mineral acids and is readily digested by amylolytic enzymes.

Mendel and Leavenworth have recently drawn the conclusion, from the examination of embryo pigs, that embryonic structures do not contain exceptionally large amounts of glycogen. The distribution of the glycogen was not observed to differ from that in the adult animal except that the liver of the embryo does not assume its glycogen-storing function early. They further draw the conclusion that the metabolic transformations of glycogen in the embryo and the adult are entirely analogous.

The lactic acid occurring in the muscular tissue of vertebrates is *paralactic* or *sarcolactic acid*,



The reaction of an inactive living muscle is alkaline, but upon the death of the muscle, or after the continued activity of a living muscle, the reaction becomes acid, due to the formation of lactic acid. There is a difference of opinion regarding the origin of this lactic acid. Some investigators claim it to arise from the carbohydrates of the muscle, while others ascribe to it a protein origin.

Among the nitrogenous extractives of muscle, those which are of the most interest in this connection are creatine and the purine bases, xanthine and hypoxanthine. Creatine is found in varying amounts in the muscles of different species, the muscles of birds having shown the largest amount. It has also been found in the blood, the brain, in transudates and in the thyroid gland. Creatine may be crystallized

and forms colorless rhombic prisms (Fig. 77, below) which are soluble in warm water and practically insoluble in alcohol and ether. Upon boiling a solution of creatine with dilute hydrochloric acid it is dehydrolyzed and its anhydride creatinine is formed. The theory that the creatine of ingested meat is transformed into creatinine and excreted in the urine has been proven untenable through the recent researches of Folin, Klercker, and Wolf and Shaffer. It is now known that under normal conditions the ingestion of creatine in no way influences

FIG. 77.—CREATINE.

the excretion of creatinine. In the case of Eck fistula dogs, however, London and Bolyarskii[1] found ingested creatine to increase the output of creatinine in the urine. This finding is of importance as throwing light upon the rôle of the liver in creatine and creatinine metabolism. In this connection it is important to note that there is no *normal* excretion of *endogenous* (see p. 267) creatine, a statement proven by the fact that if no creatine be ingested none will be excreted. Folin[2] has shown that the main bulk of ingested creatine is retained in the body, unless the diet contains a large amount of protein material. Under certain *pathological* conditions the urine may contain *endogenous* creatine which is probably derived from the catabolism of muscular tissue, as Benedict, Mellanby, and Shaffer have suggested.

Besides being a normal constituent of muscle, xanthine has been found in the brain, spleen, pancreas, thymus, kidneys, testicles, liver,

[1] London and Bolyarskii: *Zeit. phys. chem.*, LXII, p. 465, 1909.
[2] Folin: *Hammarsten Festschrift*, p. 15.

and in the urine. It may be obtained in crystalline form (Fig. 78, below), but ordinarily it is amorphous. Xanthine is easily soluble in alkalis, less soluble in water and dilute acids, and entirely insoluble in alcohol and ether.

Hypoxanthine occurs ordinarily in those tissues and fluids which contain xanthine. It has been found, unaccompanied by xanthine, in bone marrow and in milk. Unlike xanthine it may be easily crystallized in the form of small, colorless needles. It is readily soluble in alkalis, acids, and boiling water, less soluble in cold water and practically insoluble in alcohol and ether.

FIG. 78.—XANTHINE.
After the drawings of Horbaczewski, as represented in Neubauer and Vogel. (*Ogden.*)

The predominating inorganic salt of muscle is potassium phosphate. Besides this salt we have present chlorides and salts of sodium, calcium, magnesium, and iron. Sulphates are also present in *traces*.

Mendel and Saiki have recently made some interesting observations upon the chemical composition of *non-striated* (involuntary) mammalian muscle, such as the urinary bladder and the muscular coat of the stomach of the pig. Hypoxanthine was found to be the predominant purine base present. Creatine and paralactic acid were also isolated. These investigators were unable to demonstrate, definitely, the presence of glycogen in the non-striated muscles studied, but state that "the tissues possess the property of transforming glycogen in the characteristic enzymatic way." The most important part of their investigation consists in a rather complete analysis of the inorganic constituents of these muscles. A notable difference in the relative distribution of the various inorganic constituents was observed, a difference which, according to the authors, "can be accounted for in

part only by an admixture of lymph." The comparative composition of the inorganic portion of striated and non-striated muscle and of blood serum for comparison is shown in the appended table:

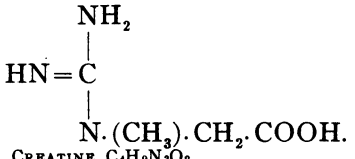
	K_2O	Na_2O	Fe_2O_3	CaO	MgO	Cl	P_2O_5	H_2O
Non-striated muscle (Mendel and Saiki)	0.081	0.328	0.011	0.044	0.007	0.171	0.184	80.6
Skeletal muscle (Katz)	0.306	0.210	0.008	0.011	0.047	0.048	0.487	72.9
Blood serum (Abderhalden)	0.027	0.425	.....	0.012	0.004	0.363	0.020	91.8

Muscular tissue is said to contain a reddish pigment called *myo-hæmatin*, which is a derivative of hæmoglobin.

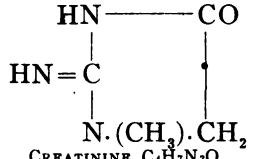
The so-called "fatigue substances" of muscle are carbon dioxide, paralactic acid, and potassium dihydrogen phosphate.

The ordinary commercial "meat extract" is composed principally of the water-soluble constituents of muscle and *contains practically nothing of nutritive value.* The protein material to which meat owes its value as an article of diet is practically all removed in the preparation of the extract.

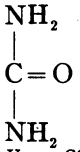
The structural formulas of the nitrogenous extractives of muscle are as follows:



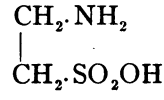
CREATINE, $C_4H_9N_3O_2$.
Methyl-guanidine acetic acid.



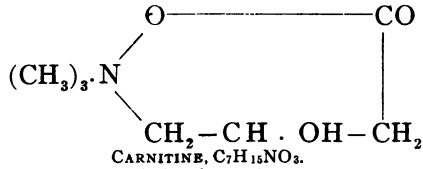
CREATININE, $C_4H_7N_3O$.
Creatine anhydride.



UREA, CON_2H_4.



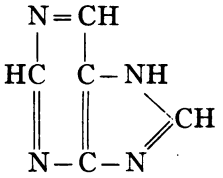
TAURINE, $C_2H_7NSO_3$.
Amino-ethyl-sulphonic acid.

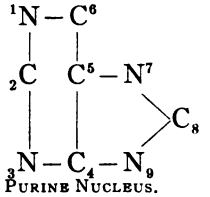


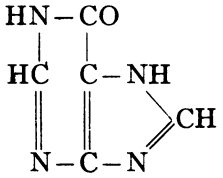
CARNITINE, $C_7H_{15}NO_3$.
γ-trimethyloxybutyrobetaine.

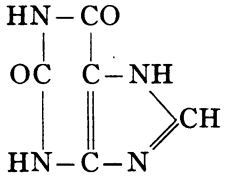
Carnosine, $C_9H_{14}N_4O_3$.
Neosine, $C_6H_{17}NO_2$.
Novaine, $C_7H_{17}NO_2$.
Ignotine, $C_9H_{14}N_4O_3$.
Phosphocarnic acid, $C_{10}H_{17}N_3O_5$ or $C_{10}H_{15}N_3O_5$.
Inosinic acid, $(HO)_2.PO.O.CH_2(CHOH)_3.CH:(C_5H_3N_4O)$.

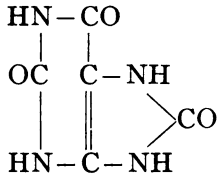
The following extractives as a group are called *purine bodies*. Their formulas, together with that of *purine* from which they are derived and the hypothetical "purine nucleus" follow:

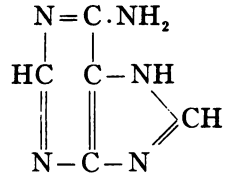

PURINE, $C_5H_4N_4$.

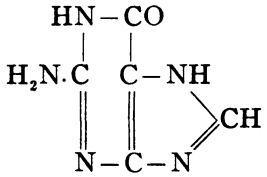

PURINE NUCLEUS.


HYPOXANTHINE, $C_5H_4N_4O$.
6-oxypurine.


XANTHINE, $C_5H_4N_4O_2$.
2–6-dioxypurine.


URIC ACID, $C_5H_4N_4O_3$.
2–6–8-trioxypurine.


ADENINE, $C_5H_5N_5$.
6-aminopurine.


GUANINE, $C_5H_5N_5O$.
2-amino–6-oxypurine.

EXPERIMENTS ON MUSCULAR TISSUE.

I. Experiments on "Living" Muscle.

1. **Preparation of Muscle Plasma (Halliburton).**—Wash out the blood vessels of a freshly killed rabbit with 0.9 per cent sodium

chloride. This can best be done by opening the abdomen and inserting a cannula into the aorta. Now remove the skin from the lower limbs, cut away the muscles and divide them into very small pieces by means of a meat chopper. Transfer the pieces of muscle to a mortar and grind them with clean sand and a little 5 per cent magnesium sulphate. Filter off the salted muscle plasma and make the following tests:

(*a*) *Reaction.*—Test the reaction to litmus. What is the reaction of this fresh muscle plasma?

(*b*) *Fractional Coagulation.*—Place a little muscle plasma in a test-tube and arrange the apparatus for fractional coagulation as explained on page 98. Raise the temperature very carefully from 30° C. and note any changes which may occur and the exact temperature at which such changes take place. When the first protein (paramyosinogen) coagulates filter it off and then heat the clear filtrate as before, being careful to note the exact temperature at which the next coagulation (myosinogen) occurs. There will probably be a preliminary opalescence in each case before the real coagulation occurs. Therefore do not mistake the real coagulation-point and filter at the wrong time. What are the coagulation temperatures of these two proteins? Which protein was present in greater amount?

(*c*) *Formation of the Myosin Clot.*—Dilute a portion of the plasma with 3 or 4 times its volume of water and place it on a water-bath or in an incubator at 35° C. for several hours. A typical *myosin clot* should form. Note the muscle serum surrounding the clot. Now test the reaction. Has the reaction changed, and if so to what is the change due? Make a test for lactic acid. What do you conclude?

2. **Preparation of Muscle Plasma (v. Furth).**—Remove the blood-free muscles of a rabbit as explained above. Finely divide by means of a meat chopper and grind in a mortar with a little clean sand and some 0.9 per cent sodium chloride. Wrap portions of the muscle in muslin and press thoroughly by means of a tincture press or lemon squeezer. Filter and make the tests according to the directions given in the last experiment.

3. **"Fuchsin-frog" Experiment.**—Inject a saturated aqueous solution of Fuchsin "S" into the lymph spaces of a frog three or four times daily for two or three days, in this way thoroughly saturating the tissues with the dye. Pith the animal (insert a heavy wire or blunt needle through the occipito atlantoid membrane), remove the skin from both hind legs and expose the sciatic nerve in one of them. Insert a small wire hook through the jaws of the frog and

suspend the animal from an ordinary clamp or iron ring. Pass electrodes under the exposed sciatic nerve, and after tying the other leg to prevent any muscular movement, stimulate the exposed nerve by means of *make* and *break* shocks from an induction coil. The stimulated leg responds by pronounced muscular contractions, whereas the tired leg remains inactive. Continue the stimulation until the muscles are fatigued. The muscular activity has caused the production of *lactic acid* and this in turn has reacted with the injected fuchsin to cause a *pink or red* color to develop. The muscles of the inactive leg still remain unchanged in color.

The normal color of the Fuchsin "S" when injected was red, but upon being absorbed it became colorless through the action of the alkalinity of the blood. Upon stimulating the muscles, however, as above explained, lactic acid was formed and this acid reacted with the fuchsin and again produced the original color of the dye.

II. Experiments on "Dead" Muscle.

1. **Preparation of Myosin.**—Take 25 grams of finely divided lean beef which has been carefully washed to remove blood and lymph constituents and place it in a beaker with 10 per cent sodium chloride. Stir occasionally for several hours. Strain off the meat pieces by means of cheese cloth, filter the solution and saturate it with sodium chloride in substance. Filter off the precipitate of *myosin* and make the tests as given below. This filtration will proceed very slowly. Myosin collects as a film on the sides of the filter paper and may be removed and tested before the entire volume of fluid has been filtered. If this precipitate remains for any length of time on the paper in contact with the air it will become transformed into the protean *myosan*. Test the myosin precipitate as follows:

(*a*) *Solubility.*—Try its solubility in the ordinary solvents. Is myosin an albumin or a globulin?

(*b*) *Xanthoproteic Reaction.*—See page 89.

(*c*) *Coagulation Test.*—Suspend a little of the myosin in water in a test-tube and heat to boiling for a few moments. Now remove the suspended material and try its solubility in 10 per cent sodium chloride. What property does this experiment show myosin to possess?

Test the filtrate from the original myosin precipitate as follows:

(*a*) *Biuret Test.*—What does this show?

(*b*) Place a little of the solution in a test-tube and heat to boiling.

At the boiling-point add a drop of dilute acetic acid and filter. Test this filtrate for proteose with picric acid. Is any proteose present? Saturate another portion of the filtrate with ammonium sulphate and test for peptone in the usual way (see page 112). Do you find any peptone? From your experiments on "living" and "dead" muscle what are your ideas regarding the proteins of muscle?

2. **Preparation of Glycogen.**—Grind a few scallops in a mortar with sand. Transfer to an evaporating dish, add water, and boil for 20 minutes. At the boiling-point faintly acidify with acetic acid. Why is this acid added? Filter, and divide the filtrate into two parts. Note the opalescence of the solution. Neutralize or make faintly alkaline one portion of the filtrate and test it as follows:

(*a*) *Iodine Test.*—To 5 c.c. of the solution in a test-tube add 5–10 drops of iodine solution and 2–3 drops of 10 per cent sodium chloride. What do you observe? Is this similar to the iodine test upon any other body with which we have had to deal?

(*b*) *Reduction Test.*—Does the solution reduce Fehling's solution?

(*c*) *Hydrolysis of Glycogen.*—Add 10 drops of concentrated hydrochloric acid to 10 c.c. of the solution and boil for 10 minutes. Cool the solution, neutralize with *solid* potassium hydroxide and test with Fehling's solution. Does it still fail to reduce Fehling's solution? If you find a reduction how can you prove the identity of the reducing substance?

(*d*) *Influence of Saliva.*—Place 5 c.c. of the solution in a test-tube, add 5 drops of saliva and place on the water-bath at 40° C. for 10 minutes. Does this now reduce Fehling's solution?

To the second part of the glycogen filtrate add 3–4 volumes of 95 per cent alcohol. Allow the glycogen precipitate to settle, decant the supernatant fluid, and filter the remainder. Heat the glycogen on a water-bath to remove the alcohol, then subject it to the following tests:

(*a*) *Solubility.*—Try its solubility in the ordinary solvents.

(*b*) *Iodine Test.*—Place a small amount of the glycogen in a depression of a test-tablet and add 2–3 drops of dilute iodine solution and a trace of a sodium chloride solution. The same wine-red color is observed as in the iodine test upon the glycogen solution.

Separation of Extractives from Muscle.

1. **Creatine.**—Dissolve about 10 grams of a commercial extract of meat in 200 c.c. of warm water. Precipitate the inorganic con-

stituents by neutral lead acetate, being careful not to add an excess of the reagent. Write the equations for the reactions taking place here. Allow the precipitate to settle, then filter and remove the excess of lead in the *warm* filtrate by hydrogen sulphide. Filter while the solution is yet warm, evaporate the clear filtrate to a syrup, and allow it to stand at least 48 hours in a cool place. Crystals of creatine should form at this point. Examine under the microscope (Fig. 77, page 234). Treat the syrup with 200 c.c. of 88 per cent alcohol, stir well with a glass rod to bring all soluble material into solution, and then filter. The purine bases have been dissolved and are in the filtrate, whereas the creatine crystals were insoluble in the 88 per cent alcohol and remain on the filter paper. Wash the crystals with 88 per cent alcohol, then remove them and bring them into solution in a little hot water. Decolorize the solution by animal charcoal and

FIG. 79.—HYPOXANTHINE SILVER NITRATE.

concentrate it to a small volume. Allow the solution to cool and note the separation of colorless crystals of creatine. Examine these crystals under the microscope and compare them with those reproduced in Fig. 77, page 234.

2. **Hypoxanthine.**—Evaporate the alcoholic filtrate from the creatine to remove the alcohol. Make the solution ammoniacal and add ammoniacal silver nitrate until precipitation ceases. The precipitate consists principally of *hypoxanthine silver* and *xanthine silver*. Collect these silver salts on a filter paper and wash them with water. Place the precipitate and paper in an evaporating dish and boil for one minute with nitric acid having a specific gravity of 1.1. Filter

while *hot* through a double paper, wash with the same strength of nitric acid and allow the solution to cool. By this treatment with nitric acid *hypoxanthine silver nitrate* and *xanthine silver nitrate* have been formed. The former is insoluble in the cold solution and separates on standing. After standing several hours filter off the hypoxanthine silver nitrate and wash with water until the wash-water is only slightly acid in reaction. Examine the crystals of *hypoxanthine silver nitrate* under the microscope and compare them with those in Fig. 79, page 241. Now wash the crystals from the paper into a beaker with a little water and warm the liquid. Remove the silver by hydrogen sulphide and filter. By this means *hypoxanthine nitrate* has been formed and is present in the filtrate. Concentrate on a water-bath to drive off hydrogen sulphide and render the solution slightly alkaline with ammonia. Warm for a time, to remove the free ammonia, filter, concentrate the filtrate to a small volume and allow it to stand in a cool place. Hypoxanthine should crystallize in small colorless needles. Examine the crystals under the microscope.

3. **Xanthine.**—To the filtrate from the above experiment containing the *xanthine silver nitrate* add ammonia in excess. (The crystalline form of xanthine silver nitrate is shown in Fig. 80, below.)

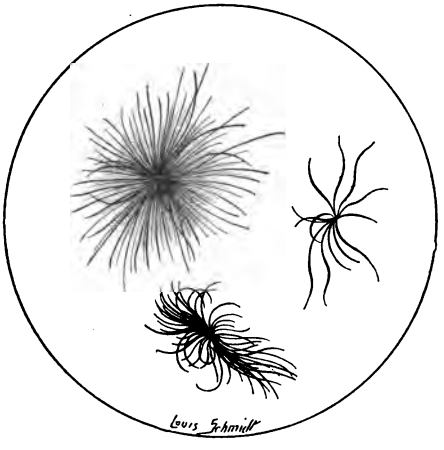


FIG. 80.—XANTHINE SILVER NITRATE.

A brownish-red precipitate of *xanthine silver* forms. Treat this suspended precipitate with hydrogen sulphide (do not use an excess of hydrogen sulphide), warm the mixture for a few moments and filter while hot. Concentrate the filtrate to a small volume and put away in a cool place for crystallization (Fig. 78, p. 235). To obtain xanthine in crystalline form special precautions are generally necessary.

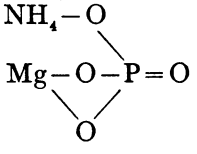
Evaporate the solution to dryness. Make the following tests on the crystals or residue:

(*a*) *Xanthine Test.*—Place about one-half of the crystalline or amorphous material in a small evaporating dish, add a few drops of concentrated nitric acid and evaporate to dryness very carefully on a water-bath. The yellow residue upon moistening with caustic potash becomes red in color and upon further heating assumes a purplish-red hue. Now add a few drops of water and warm. In this way a yellow solution results which yields a red residue upon evaporation. How does this differ from the Murexide test upon uric acid?

(*b*) *Weidel's Reaction.*—By gently heating bring the remainder of the xanthine crystals or residue into solution in bromine-water. Evaporate the solution to dryness on a water-bath. Remove the stopper from an ammonia bottle and by blowing across the mouth of the bottle direct the fumes of ammonia so that they come in contact with the dry residue. Under these conditions the presence of xanthine is shown by the residue assuming a red color. A somewhat brighter color may be obtained by using a trace of nitric acid with the bromine-water. By the use of this modification, however, we may get a positive reaction with bodies other than xanthine.

HÜRTHLE'S EXPERIMENT.

Tease a very small piece of frog's muscle on a microscopical slide. Expose the slide to ammonia vapor for a few moments, then adjust a coverglass, and examine the muscle fibers under the microscope. Note the large number of crystals of ammonium magnesium phosphate,



distributed everywhere throughout the muscle fiber, thus demonstrating the abundance of phosphates and magnesium in the muscle (Fig. 96, page 296.)

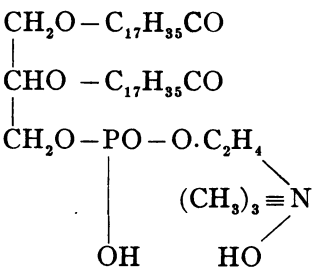
CHAPTER XVI.

NERVOUS TISSUE.

In common with the other solid tissues of the body, nervous tissue contains a large amount of water. The percentage of water present depends upon the particular form of nervous tissue but in all forms it is invariably greater in the gray matter than in the white. Embryonic nervous tissues also contain a larger percentage of water than the tissues of adult life. The gray matter of the brain of the fœtus, for instance, contains about 92 per cent of water, whereas the gray matter of the brain of the adult contains but 83–84 per cent of the fluid.

Among the solid constituents of nervous tissue are *proteins, cholesterol, cerebrin, lecithin, kephalin, protagon (?), paranucleoprotagon, nuclein, neurokeratin, collagen, extractives,* and *inorganic salts.* The proteins are present in the greatest amount and comprise about 50 per cent of the total solids. Three distinct proteins, two globulins, and a nucleoprotein, have been isolated from nervous tissue. The globulins coagulate at 47° C. and 70–75° C., respectively, while the nucleoprotein coagulates at 56–60° C. This nucleoprotein contains about 0.5 per cent of phosphorus (Halliburton, Levene). Nervous tissue is composed of a relatively large quantity of a variety of compounds which collectively may be grouped under the term "lipoid"—substances resembling the fats in some of their physical properties and reactions but distinct in their composition. We will class cerebrin, cholesterol, and the phosphorized fats as "lipoids."

The group of *phosphorized fats* are very important constituents of nervous tissue. The best known members of this group are *lecithin, protagon (?)* and *kephalin.* Lecithin occurs in larger amount than the other members of the group, has been more thoroughly studied than the others and is apparently of greater importance. Upon decomposition lecithin yields *fatty acid, glycero-phosphoric acid,* and *choline.* Each lecithin molecule contains two fatty acid radicals which may be those of the same or different fatty acids. Thus we have different lecithins depending upon the particular fatty acid radicals which are present in the molecule. The formula of a typical lecithin would be the following:

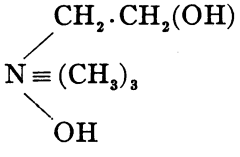


This lecithin would be called distearyl-lecithin or *choline-distearyl-glycero-phosphoric acid.* Upon decomposition the molecule splits according to the following reaction:

$$\underset{\text{Lecithin.}}{C_{44}H_{90}NPO_9} + 3H_2O = 2\underset{\text{Stearic acid.}}{(C_{18}H_{36}O_2)} + \underset{\text{Glycero-phosphoric acid.}}{C_3H_9PO_6} + \underset{\text{Choline.}}{C_5H_{15}NO_2}.$$

The lecithins are not confined to the nervous tissues but are found in nearly all animal and vegetable tissues. Lecithin is a primary constituent of the cell. It is soluble in chloroform, ether, alcohol, benzene, and carbon disulphide. The chloroform or alcohol-ether solution may be precipitated by acetone. Lecithin may be caused to crystallize in the form of small plates by cooling the alcoholic solution to a low temperature. It has the power of combining with acids and bases, and the hydrochloric acid combination has the power of forming a double salt with platinic chloride.

Choline, as was indicated above, is one of the decomposition products of lecithin. It is *trimethyl-oxyethyl-ammonium hydroxide* and has the following formula:



Recent researches have shown that great importance is to be attached to the detection of choline in the cerebro-spinal fluid and the blood in certain cases of degenerative disease of the nervous system. In this connection tests for choline (see p. 248) are of interest and value.

Protagon, another nitrogenous phosphorized substance is a body over which there has been much discussion. Upon decomposition it is said by some investigators to yield cerebrin and the decomposition

products of lecithin. It has recently been shown by Posner and Gies as well as by Rosenheim and Tebb that protagon is a mixture and has no existence as a chemical individual.

Kephalin is the third member of the group of phosphorized fats. It is precipitated from its acetone-ether extract by alcohol. It contains about 4 per cent of phosphorus and has been given the formula $C_{42}H_{79}NPO_{13}$. Kephalin may be a stage in lecithin metabolism.

Cerebrin, a substance containing nitrogen but no phosphorus, is an important constituent of the white matter of nervous tissue. It has also been found in the spleen, pus, and in egg yolk. It may be extracted from the tissue by boiling alcohol and is insoluble in cold alcohol, cold and hot ether, and in water and dilute alkalis. Cerebrin is a mixture containing phrenosin (pseudo-cerebrin or cerebron), a body yielding the carbohydrate galactose on decomposition.

Cholesterol, one of the primary cell constituents, is present in fairly large amount in nervous tissue. It is a mon-atomic alcohol with the formula $C_{27}H_{45}OH$. It was formerly called a "non-saponifiable fat" but since it is not changed in any way by boiling alkalis it is not a fat. It is soluble in ether, chloroform, benzene, and hot alcohol. It crystallizes in the form of thin, colorless, transparent plates (Fig. 42, p. 155). Cholesterol occurs abundantly in one form of biliary calculus. It has also been found in feces, wool fat, egg yolk, and milk, frequently in the form of its esters of higher fatty acids.

Paranucleoprotagon is a phosphorized substance originally isolated from brain tissue by Ulpiani and Lelli and recently reinvestigated by Steel and Gies. It is said to possess lecithoprotein characteristics.

Nervous tissue yields about 1 per cent of ash which is made up in great part of alkaline phosphates and chlorides.

Experiments on the Lipoids of Nervous Tissue.[1]

1. **Preparation of Lecithin.**—Treat the macerated brain of a sheep with ether and allow it to stand in the cold for 48–72 hours. The cold ether will extract lecithin and cholesterol. Filter and add acetone to the filtrate to precipitate the lecithin. Filter off the lecithin and test it as follows:

[1] Preparation of So-called Protagon.—Macerate the brain of a sheep, treat with 85 per cent alcohol and warm on a water-bath at 45° C. for two hours. Filter *hot* into a bottle or strong flask and cool to 0° C. for one-half hour by means of a freezing mixture. By this procedure both protagon and cholesterol are caused to precipitate. Filter the cold solution rapidly and treat the precipitate on the paper with ice cold ether to dissolve out the cholesterol. The protagon may now be redissolved in warm 85 per cent. alcohol from which solution it will precipitate upon cooling.

(*a*) *Microscopical Examination.*—Suspend a small portion in a drop of water on a slide and examine under the microscope.

(*b*) *Osmic Acid Test.*—Treat a small portion with osmic acid. What happens?

(*c*) *Acrolein Test.*—Make the acrolein test according to directions on page 132.

(*d*) *"Fusion" Test for Phosphorus.*—Place some of the lecithin prepared above in a small porcelain crucible, add a suitable amount of a fusion mixture composed of potassium hydroxide and potassium nitrate (5 : 1) and heat *carefully* until the resulting mixture is colorless. Cool, dissolve the mass in a little warm water, acidify with nitric acid, heat to boiling, and add a few cubic centimeters of molybdic solution. In the presence of phosphorus a yellow precipitate forms. What is it?

2. **Preparation of Cholesterol.**—Place a small amount of macerated brain tissue under ether and stir occasionally for one hour. Filter, evaporate the filtrate to dryness on a water-bath, and test the cholesterol according to directions given below. (If it is desired, the ether extract from the so-called protagon, or the ether-acetone filtrate from the lecithin may be used for the isolation of cholesterol. In these cases it is simply necessary to evaporate the solution to dryness on a water-bath.) Upon the cholesterol prepared by either of the above methods make the following tests:

(*a*) *Microscopical Examination.*—Examine the crystals under the microscope and compare them with those in Fig. 42, page 155.

(*b*) *Iodine-sulphuric Acid Test.*—Place a few crystals of cholesterol in one of the depressions of a test-tablet and treat with a drop of concentrated sulphuric acid and a drop of a very dilute solution of iodine. A play of colors, consisting of violet, blue, green, and red, results.

(*c*) *The Liebermann-Burchard Test.*—Dissolve a few crystals of cholesterol in 2 c.c. of chloroform in a *dry* test-tube. Now add 10 drops of acetic anhydride and 1–3 drops of concentrated sulphuric acid. The solution becomes red, then blue, and finally bluish-green in color.

(*d*) *Salkowski's Test.*—Dissolve a few crystals of cholesterol in a little chloroform and add an equal volume of concentrated sulphuric acid. A play of colors from bluish-red to cherry-red and purple is noted in the chloroform, while the acid assumes a marked green fluorescence.

(*e*) *Schiff's Reaction.*—To a little cholesterol in an evaporating

dish add a few drops of Schiff's reagent.[1] Evaporate to dryness over a low flame and observe the reddish-violet residue which changes to a bluish-violet.

(*f*) *Phosphorus.*—Test for phosphorus according to directions given on page 247. Is phosphorus present?

3. **Preparation of Cerebrin.**—Treat the macerated brain tissue, in a flask, with 95 per cent alcohol and boil on a water-bath for one-half hour, keeping the volume constant by adding fresh alcohol as needed. Filter the solution *hot* and stand the *cloudy* filtrate away for twenty-four hours. (If the filtrate is not cloudy concentrate it upon the water-bath until it is so.) Filter off the cerebrin and test it as follows:

(*a*) *Microscopical Examination.*—Suspend a small portion in a drop of water on a slide and examine under the microscope.

(*b*) *Solubility.*—Try the solubility of cerebrin in the usual solvents and in hot and cold alcohol and hot and cold ether.

(*c*) *Phosphorus.*—Test for phosphorus according to directions on page 247. How does the result compare with that on lecithin?

(*d*) Place a little cerebrin on platinum foil and warm. Note the odor.

(*e*) *Hydrolysis of Cerebrin.*—Place the remaining cerebrin in a small evaporating dish, add equal volumes of water and dilute hydrochloric acid, and boil for one hour. Cool, neutralize with *solid* potassium hydroxide, filter, and test with Fehling's solution. Is there any reduction, and if so how do you explain it?

4. **Tests for Choline.**—(*a*) *Rosenheim's Periodide Test.*—Prepare an alcoholic extract of the fluid under examination, and after evaporation, apply Rosenheim's iodo-potassium iodide solution[2] to a little of the residue. In a short time dark brown plates and prisms of *choline periodide* begin to form and may be detected by means of the microscope. Occasionally they are large enough to be visible to the naked eye. They somewhat resemble crystals of hæmin (see p. 194). If the slide be permitted to stand, thus allowing the fluid to evaporate, the crystals will disappear and leave brown oily drops. They will reappear, however, upon the addition of fresh iodine solution. v. Staněk claims that this choline compound has the formula $C_5H_{14}NOI.I_8$.

(*b*) *Rosenheim's Bismuth Test.*—Extract the fluid under exam-

[1] Schiff's reagent consists of a mixture of three volumes of concentrated sulphuric acid and one volume of 10 per cent ferric chloride.

[2] Prepared by dissolving 2 grams of iodine and 6 grams of potassium iodide in 100 c.c. of water.

ination with absolute alcohol, evaporate, and re-extract the residue. Repeat the extraction several times. Dissolve the final residue in 2–3 c.c. of water and add a drop of Kraut's reagent.[1] Choline is indicated by the appearance of a bright brick-red precipitate.

[1] Dissolve 272 grams of potassium iodide in water and add 80 grams of bismuth subnitrate dissolved in 200 grams of nitric acid (sp. gr. 1.18). Permit the potassium nitrate to crystallize out, then filter it off and make the filtrate up to 1 liter with water.

+ Millon
+ Biuret.

CHAPTER XVII.

URINE: GENERAL CHARACTERISTICS OF NORMAL AND PATHOLOGICAL URINE.

Volume.—The volume of urine excreted by normal individuals during any definite period fluctuates within very wide limits. The average output for twenty-four hours is placed by German writers between 1500 and 2000 c.c. This value is not strictly applicable to conditions in America, however, since it has been found that the average normal excretion of the adult male American falls within the lower values of 1000–1200 c.c. The volume-excretion is influenced greatly by the diet, particularly by the ingestion of fluids.

Certain pathological conditions cause the output of urine for any definite period to depart very decidedly from the normal output. Among the pathological conditions in which the volume of urine is *increased* above normal are the following: Diabetes mellitus, diabetes insipidus, certain diseases of the nervous system, contracted kidney, amyloid degeneration of the kidney, and in convalescence from acute diseases in general. Many drugs such as calomel, digitalis, acetates, and salicylates also increase the volume of the urine excreted. A *decrease* from the normal is observed in the following pathological conditions: Acute nephritis, diseases of the heart and lungs, fevers, diarrhœa, and vomiting.

Color.—Normal urine ordinarily possesses a yellow tint, the depth of the color being dependent in part upon the density of the fluid. The color of normal urine is due principally to a pigment called *urochrome:* traces of *hæmatoporphyrin, urobilin,* and *uroerythrin* have also been detected. Under pathological conditions the urine is subject to pronounced variations in color and may contain many varieties of pigments. Under such circumstances the urine may vary in color from an extremely light yellow to a very dark brown or black. Vogel has constructed a color chart which is of some value for purposes of comparison. The nature and origin of the chief variations in the urinary color are set forth in tabular form by Halliburton as follows:

Color.	Cause of Coloration.	Pathological Condition.
Nearly colorless.....	Dilution, or diminution of normal pigments.	Nervous conditions: hydruria, diabetes insipidus, granular kidney.
Dark yellow to brown-red	Increase of normal, or occurrence of pathological, pigments.	Acute febrile diseases.
Milky	Fat globules	Chyluria.
	Pus corpuscles	Purulent diseases of the urinary tract.
Orange	Excreted drugs	Santonin, chrysophanic acid.
Red or reddish	Hæmatoporphyrin ..... Unchanged hæmoglobin....	Hæmorrhages, or hæmoglobinuria.
	Pigments in food (logwood, madder, bilberries, fuchsin).	
Brown to brown-black	Hæmatin	Small hæmorrhages.
	Methæmoglobin	Methæmoglobinuria.
	Melanin	Melanotic sarcoma.
	Hydrochinon and catechol..	Carbolic-acid poisoning.
Greenish-yellow, greenish-brown, approaching black.	Bile-pigments	Jaundice.
Dirty green[1] or blue	A dark-blue scum on surface, with a blue deposit, due to an excess of indigo-forming substances.	Cholera, typhus; seen especially when the urine is putrefying.
Brown-yellow to red-brown, becoming blood-red upon adding alkalis.	Substances contained in senna, rhubarb, and chelidonium which are introduced into the system.	

Transparency.—Normal urine is ordinarily perfectly clear and transparent when voided. On standing for a variable time, however, a cloud (nubecula) consisting principally of nucleoprotein or mucoid (see p. 284) and epithelial cells forms. A turbidity due to the precipitation of phosphates is normally noted in urine passed after a hearty meal. The urine obtained 2–3 hours after a meal or later is ordinarily free from turbidity. Permanently turbid urines ordinarily arise from pathological conditions.

[1] This dirty green or blue color also occurs after the use of methylene blue in the organism.

Odor.—The odor of normal urine is of a faint, aromatic type. The bodies to which this odor is due are not well known, but it is claimed by some investigators to be due, at least in part, to the presence of minute amounts of certain volatile organic acids. When the urine undergoes decomposition, *e. g.*, in alkaline fermentation, a very unpleasant ammoniacal odor is evolved. All urines are subject to such decomposition if allowed to stand for a sufficiently long time. Under normal conditions the urine very often possesses a peculiar odor due to the ingestion of some certain drug or vegetable. For instance, cubebs, copaiba, myrtol, saffron, tolu, and turpentine each imparts a somewhat specific odor to the urine. After the ingestion of asparagus, the urine also possesses a typical odor.

Frequency of Urination.—The frequency of urination varies greatly in different individuals but in general is dependent upon the amount of fluid in the bladder. In pathological conditions an inflammatory affection of the urinary tract or any disturbance of the innervation of the bladder will influence the frequency. Affections of the spinal cord which lead to an increased irritability of the bladder or a weakening of the sphincter will result in increasing the frequency of urination.

Reaction.—The mixed twenty-four hour urinary excretion of a normal individual ordinarily possesses an acid reaction to litmus. This acidity is now believed to be due to the presence of various acidic radicals and not to the presence of *sodium di-hydrogen phosphate* as was formerly held (see Phosphates, p. 294). This conclusion is reinforced by the observation that urine may be divided into two portions, one part consisting almost entirely of inorganic matter, including practically *all of the phosphates* and having an *alkaline reaction*, the other containing practically all of the *organic substances* and no phosphates and having an *acid reaction*. The acidity imparted to the urine by any particular acid depends entirely upon the extent to which the acid is dissociable, since it is the hydrogen ion which is responsible for the acid reaction.

The composition of the food is perhaps the most important factor in determining the reaction of the urine. The reaction ordinarily varies considerably according to the time of day the urine is passed. For instance, for a variable length of time after a meal the urine may be neutral or even alkaline in reaction to litmus, owing to the claim of the gastric juice upon the acidic radicals to further the formation of hydrochloric acid for use in carrying out the digestive secretory function. This change in reaction is known as the *alkaline tide* and is common to

perfectly healthy individuals. The urine may also become temporarily alkaline in reaction to litmus, as the result of ingesting alkaline carbonates or certain salts of tartaric and citric acids which may be trans-

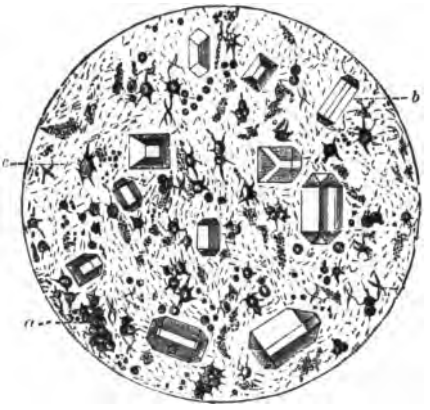


FIG. 81.—DEPOSIT IN AMMONIACAL FERMENTATION.
a, Acid ammonium urate; *b*, ammonium magnesium phosphate; *c*, bacteria.

formed into carbonates within the organism. Normal urine upon standing for some time becomes alkaline in reaction to litmus, owing to the inception of alkaline or ammoniacal fermentation through the agency of micro-organisms. This fermentation has no especial diagnostic value except in cases where the urine has undergone this change

FIG. 82.—DEPOSIT IN ACID FERMENTATION.
a, Fungus, *b*, amorphous sodium urate; *c*, uric acid; *d*, calcium oxalate.

within the organism and is voided in the decomposed state. Ammoniacal fermentation is ordinarily due to cystitis or occurs as the result of infection in the process of catheterization. A microscopical examina-

tion of such urine (Fig. 81, p. 253) shows the presence of *ammonium magnesium phosphate* crystals, *amorphous phosphates*, and not infrequently *ammonium urate*.

Occasionally a urine which possesses a normal acidity when voided, upon standing instead of undergoing ammoniacal fermentation as above described will become still more strongly acid in reaction. Such a phenomenon is termed *acid fermentation*. Accompanying this increased acidity there is ordinarily a deepening of the tint of the urinary color. Such urines may contain *acid urates*, *uric acid*, *fungi*, and *calcium oxalate* (Fig. 82, p. 253). On standing for a sufficiently long time any urine which exhibits acid fermentation will ultimately change in reaction, due to the inception of alkaline fermentation, and will show the microscopical deposits characteristic of such a urine.

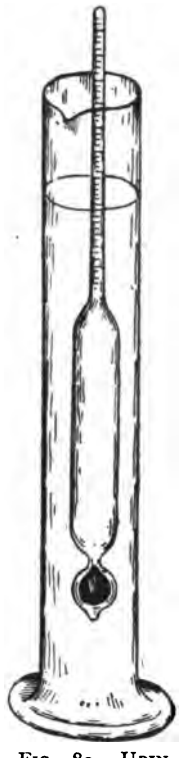


FIG. 83.—URINOMETER AND CYLINDER.

Specific Gravity.—The specific gravity of the urine of normal individuals varies ordinarily between 1.015 and 1.025. This value is subject to wide fluctuations under various conditions. For instance, following copious water- or beer-drinking the specific gravity may fall to 1.003 or lower, whereas in cases of excessive perspiration it may rise as high as 1.040 or even higher. Where a very accurate determination of thc specific gravity is desired use is commonly made of the *pyknometer* or of the *Westphal hydrostatic balance*. These instruments, however, are not suited for clinical use. The clinical method of determining the specific gravity is by means of a *urinometer* (Fig. 83,). This affords a very rapid method and at the same time is sufficiently accurate for clinical purposes. The urinometer is always calibrated for use at a specific temperature and the observations made at any other temperature must be subjected to a certain correction to obtain the true specific gravity. In making this correction *one unit of the last order is added* to the observed specific gravity for every three degrees *above* the normal temperature and *subtracted* for every three degrees *below* the normal temperature. For instance, if in using a urinometer calibrated for 15° C. the specific gravity of a urine having a temperature of 21° C. is determined as 1.018 it is necessary to add to the observed specific gravity two units of the third order to obtain

the real specific gravity of the urine. Therefore the true specific gravity, at 15° C., of a urine having a specific gravity of 1.018 at 21° C. is 1.018+0.002=1.020.

Pathologically, the specific gravity may be subjected to very wide variations. This is especially true in diseases of the kidneys. In acute nephritis ordinarily the urine is concentrated and of a high specific gravity, whereas in chronic nephritis the reverse conditions are more apt to prevail. In fact, under most conditions, whether physiological or pathological, the specific gravity of the urine is inversely proportional to the volume excreted. This is not true of diabetes mellitus, however, where the volume of urine is large and the specific gravity is also high, owing to the sugar contained in the urine.

The amount of solids eliminated in the excretion for twenty-four hours may be roughly calculated by means of *Long's coefficient, i. e.*, 2.6. The solid content of 1000 c.c. of urine is obtained by multiplying the last two figures of the specific gravity observed at 25° C. by 2.6. To determine the amount of solids excreted in twenty-four hours if the volume was 1120 c.c. and the specific gravity was 1.018 the calculation would be as follows:

(*a*) $18 \times 2.6 = 46.8$ grams of solid matter in 1000 c.c. of urine.

(*b*) $\frac{46.8 \times 1120}{1000} = 52.4$ grams of solid matter in 1120 c.c. of urine.

The coefficient of Häser (2.33) which has been in use for years probably gives values that are inaccurate for conditions existing in America. This coefficient was calculated on the basis of the specific gravity determined at a temperature of 15° C.

Freezing-point (Cryoscopy).—The freezing-point of a solution depends upon the total number of molecules of solid matter dissolved in it. The determination of the osmotic pressure by this method has recently come to be of some clinical importance, particularly as an aid in the diagnosis of kidney disorders. In this connection it is best to collect the urine from each kidney separately and determine the freezing-point in the individual samples so collected. By this means considerable aid in the diagnosis of renal diseases may be secured. The fluids most frequently examined cryoscopically are the blood (see p. 178) and the urine. The freezing-point is denoted by Δ. The value of Δ for normal urine varies ordinarily between —1.3° and —2.3° C., the freezing-point of pure water being taken as 0°. Δ is subject to very wide fluctuations under unusual conditions. For instance, following copious water- or beer-drinking Δ may have as high a value as —0.2° C., whereas on a diet containing much salt and deficient in fluids

the value of Δ may be lowered to —3° C. or even lower. The freezing-point of normal blood is generally about —0.56° C. and is not subject to the wide variations noted in the urine, because of the tendency of the organism to maintain the normal osmotic pressure of the blood under all conditions. Variations between —0.51° and —0.62° C. may be due entirely to dietary conditions, but if any marked variation is noted it can, in most cases, be traced to a disordered kidney function.

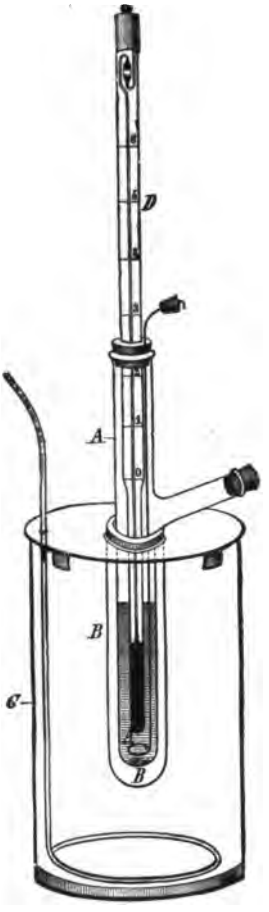


FIG. 84.—BECKMANN-HEIDENHAIN FREEZING-POINT APPARATUS. (*Long.*)

D, a delicate thermometer; *C*, the containing jar; *B*, the outside or air mantle tube; *A*, the tube in which the mixture to be observed is placed. Two stirrers are shown, one for the cooling mixture in the jar and one for the experimental mixture.

Freezing-point determinations may be made by means of the Beckmann-Heidenhain apparatus (Fig. 84) or the Zikel pektoscope. The Beckmann-Heidenhain apparatus consists of the following parts: A strong battery jar or beaker (C) furnished with a metal cover which is provided with a circular hole in its center. This strong glass vessel serves to hold the freezing mixture by means of which the temperature of the fluid under examination is lowered. A large glass tube (B) designed as an air-jacket, and formed after the manner of a test-tube is introduced through the central aperture in the metal cover and into this air-jacket is lowered a smaller tube (A) containing the fluid to be tested. A very delicate thermometer (D), graduated in hundredths of a degree is introduced into the inner tube and is held in place by means of a cork so that the mercury bulb is immersed in the fluid under examination but does not come in contact with any glass surface. A small platinum wire stirrer serves to keep the fluid under examination well mixed while a larger stirrer is used to manipulate the freezing mixture. (Rock salt and ice in the proportion 1 : 3 form a very satisfactory freezing mixture.)

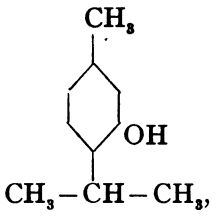
In making a determination of the freezing-point of a fluid by means of the Beckmann-Heidenhain apparatus proceed as follows: Place the freezing mixture in the battery jar and add water (if necessary) to secure

a temperature not lower than 3° C. Introduce the fluid to be tested into tube A, place the thermometer and platinum wire stirrer in position, and insert the tube into the air-jacket which has previously been inserted through the metal cover of the battery jar. Manipulate the two stirrers in order to insure an equalization of temperature and observe the course of the mercury column of the thermometer very carefully. The mercury will *gradually fall* and this gradual lowering of the temperature will be followed by a *sudden rise*. The point at which the mercury rests *after this sudden rise* is the *freezing-point*. This rise is due to the fact that previous to freezing, a fluid is always more or less *overcooled* and the thermometer temporarily registers a temperature somewhat *below the freezing-point*. As the fluid freezes, however, there is a very *sudden* change in the temperature of the liquid and this change is imparted to the thermometer and causes the rise as indicated. It occasionally occurs that the fluid under examination is very much overcooled and *does not freeze*. Under such circumstances a small piece of ice is introduced into it by means of the side tube noted in the figure. This so-called "inoculation" causes the fluid to freeze instantaneously. (For details of the method of determining the freezing-point consult standard works on physical or organic chemistry.)

Electrical Conductivity.—The electrical conductivity of the urine is dependent upon the number of *inorganic* molecules or ions present, and in this differs from the freezing-point which is dependent upon the total number of molecules both *inorganic* and *organic* which are in solution. The conductivity of the urine has been investigated but slightly, and this very recently, but from the data secured it seems that the value generally falls below $\kappa = 0.03$. The conductivity of blood serum has been determined as $\kappa = 0.012$. Up to the present time the determination of the electrical conductivity of any of the fluids of the body has been put to very slight clinical use. Experience may show the conductivity value to be a more important aid to diagnosis than it is now considered, particularly if it is taken in connection with the determination of the freezing-point. By a combination of these two methods the portion of the osmotic pressure due respectively to electrolytes and non-electrolytes may be determined. For a discussion of electrical conductivity, the method by which it is determined, and the principles involved consult standard works on physical or electrochemistry.

Collection of the Urine Sample.—If any dependable data are desired regarding the *quantitative* composition of the urine the examination of the mixed excretion for twenty-four hours is *absolutely necessary*.

In collecting the urine the bladder may be emptied at a given hour, say 8 A. M., the urine discarded and all the urine from that hour up to and including that passed the next day at 8 A. M., saved, thoroughly mixed, and a sample taken for analysis. Powdered thymol,



is a very satisfactory preservative since the excess may be removed by filtration, if desired, and any small amount which may go into solution will have no appreciable influence upon the determination of any of the urinary constituents. It has no reducing power and so may safely be used to preserve diabetic urines. To insure the preservation of the mixed urine of the twenty-four hour period it is advisable to place a small amount of the thymol powder in the urine receptacle before the first fraction of urine is voided. In order to further insure the preservation of the urine the cleaned and dried urine receptacle may be rinsed with an alcoholic solution of thymol and subsequently thoroughly dried before introducing the urine.

Toluene is also used for the preservation of urine.

In certain pathological conditions it is desirable to collect the urine passed during the *day* separately from that passed during the *night.* When this is done the urine voided between 8 A. M. and 8 P. M. may be taken as the *day sample* and that voided between 8 P. M. and 8 A. M. as the *night sample.*

The *qualitative* testing of urine voided at *random*, except in a few specific instances, is of no particular value so far as giving us any accurate knowledge as to the exact urinary characteristics of the individual is concerned. In the great majority of cases the qualitative as well as the quantitative tests should be made upon the mixed excretion for a twenty-four hour period.

CHAPTER XVIII.

URINE: PHYSIOLOGICAL CONSTITUENTS.[1]

1. Organic Physiological Constituents.

Urea.
Uric acid.
Creatinine.
Creatine.

Ethereal sulphuric acids.....
- Indoxyl-sulphuric acid.
- Phenol- and *p*-cresol-sulphuric acids.
- Pyrocatechin-sulphuric acid.
- Skatoxyl-sulphuric acid.

Hippuric acid.
Oxalic acid.

Neutral sulphur compounds.
- Cystine.
- Chondroitin-sulphuric acid.
- Thiocyanates.
- Taurine derivatives.
- Oxyproteic acid.
- Alloxyproteic acid.
- Uroferric acid.

Allantoïn.

Aromatic oxyacids.....
- Paraoxyphenyl-acetic acid.
- Paraoxyphenyl-propionic acid.
- Homogentisic acid.
- Uroleucic acid.
- Oxymandelic acid.
- Kynurenic acid.

Benzoic acid.
Neucleoprotein.
Oxaluric acid.

[1] It is impossible to make any *absolute* classification of the physiological and pathological constituents of the urine. A substance may be present in the urine in small amount physiologically and be sufficiently increased under certain conditions as to be termed a pathological constituent. Therefore it depends, in some instances, upon the *quantity* of a constituent present whether it may be correctly termed a physiological or a pathological constituent.

Enzymes	Pepsin.
	Gastric rennin.
	Amylase.
Volatile fatty acids	Acetic acid.
	Butyric acid.
	Formic acid.
Paralactic acid.	
Phenaceturic acid.	
Phosphorized compounds	Glycerophosphoric acid.
	Phosphocarnic acid.
Pigments	Urochrome.
	Uroblin.
	Uroerythrin.
Ptomaines and leucomaines.	
Purine Bases	Adenine.
	Guanine.
	Xanthine.
	Epiguanine.
	Episarkine.
	Hypoxanthine.
	Paraxanthine.
	Heteroxanthine.
	1-Methylxanthine.

2. Inorganic Physiological Constituents.

Ammonia.
Sulphates.
Chlorides.
Phosphates.
Sodium and potassium.
Calcium and magnesium.
Carbonates.
Iron.
Fluorides.
Nitrates.
Silicates.
Hydrogen peroxide.

UREA, $\begin{matrix} NH_2 \\ | \\ C=O. \\ | \\ NH_2 \end{matrix}$

Urea is the principal end-product of the metabolism of protein substances. It has been generally believed that about 90 per cent of the total nitrogen of the urine was present as urea. Recently, however, Folin has shown that the distribution of the nitrogen of the urine among urea and the other nitrogen-containing bodies present depends entirely upon the absolute amount of the total nitrogen excreted. He found that a decrease in the total nitrogen excretion was always accompanied by a decrease in the percentage of the total nitrogen excreted as urea, and that after so regulating the diet of a

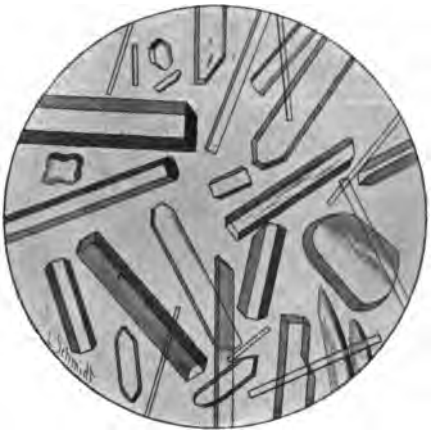


FIG. 85.—UREA.

normal person as to cause the excretion of total nitrogen to be reduced to 3–4 grams in 24 hours, *only about 60 per cent of this nitrogen appeared in the urine as urea.* His experiments also seem to show urea to be the only one of the nitrogenous excretions which is relatively as well as absolutely decreased as a result of decreasing the amount of protein metabolized. This same investigator reports a hospital case in which only 14.7 per cent of the total nitrogen was present as urea and about 40 per cent was present as ammonia. Mörner had previously reported a case in which but 4.4 per cent of the total nitrogen of the urine was present as urea, and 26.7 per cent was present as ammonia.

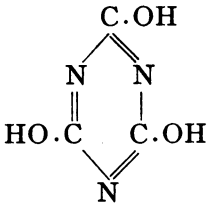
Urea occurs most abundantly in the urine of man and carnivora and in somewhat smaller amount in the urine of herbivora; the urine of fishes, amphibians, and certain birds also contain a small amount of the substance. Urea is also found in nearly all the fluids and in many of the tissues and organs of mammals. The amount excreted, under normal conditions, by an adult man in 24 hours is about 30 grams; women excrete a somewhat smaller amount. The excretion is greatest

in amount after a diet of meat, and least in amount after a diet consisting of non-nitrogenous foods; this is due to the fact that the last-mentioned diet has a tendency to decrease the metabolism of the tissue proteins and thus cause the output of urea under these conditions to fall below the output of urea observed during starvation. The output of urea is also increased after copious water- or beer-drinking. The increase is probably due *primarily* to the washing out of the tissues of the urea previously formed, but which had not been removed in the normal processes, and *secondarily* to a stimulation of protein catabolism.

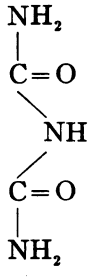
Urea may be formed in the organism from amino acids such as leucine, glycocoll, and aspartic acid: it may also be formed from ammonium carbonate $(NH_4)_2CO_3$ or ammonium carbamate, $H_4N.O.CO.NH_2$.

There are differences of opinion regarding the transformation of the substances just named into urea, but there is rather conclusive evidence that at least a part of the urea is formed in the liver; it may be formed in other organs or tissues as well.

Urea crystallizes in long, colorless, four- or six-sided, anhydrous, rhombic prisms (Fig. 85, p. 261), which melt at 132° C. and are soluble in water or alcohol and insoluble in ether or chloroform. If a crystal of urea is heated in a test-tube, it melts and decomposes with the liberation of ammonia. The residue contains *cyanuric acid*,



and *biuret*,



The biuret may be dissolved in water and a reddish-violet color obtained by treating the aqueous solution with cupric sulphate and potassium hydroxide (see Biuret Test, p. 90). Certain hypochlorites

or hypobromites in alkaline solution have the power of decomposing urea into nitrogen, carbon dioxide, and water. Sodium hypobromite brings about this decomposition, as follows:

$$CO(NH_2)_2 + 3NaOBr = 3NaBr + N_2 + CO_2 + 2H_2O.$$

This property forms the basis for a clinical quantitative determination of urea (see page 369).

Urea has the power of forming crystalline compounds with certain acids; urea nitrate and urea oxalate are the most important of these compounds. *Urea nitrate*, $CO(NH_2)_2.HNO_3$, crystallizes in colorless, rhombic or six-sided tiles (Fig. 86, below), which are easily soluble in water. *Urea oxalate*, $2.CO(NH_2)_2.H_2C_2O_4$, crystallizes in the form of rhombic or six-sided prisms or plates (Fig. 88, p. 265): the oxalate differs from the nitrate in being somewhat less soluble in water.

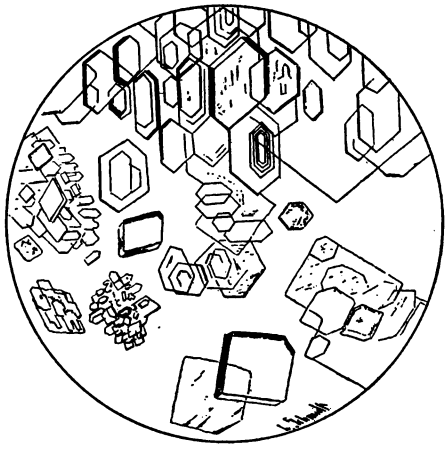


FIG. 86.—UREA NITRATE.

A decrease in the excretion of urea is observed in many diseases in which the diet is much reduced and in some disorders as a result of alterations in metabolism, *e. g.*, myxœdema, and in others as a result of changes in excretion, as in severe and advanced kidney disease. A pathological increase is found in a large proportion of diseases which are associated with a toxic state.

EXPERIMENTS ON UREA.

1. **Isolation from the Urine.**—Place 800 c.c. of urine in a precipitating jar, add 250 c.c. of baryta mixture,[1] and stir thoroughly.

[1] Baryta mixture consists of a mixture of one volume of a saturated solution of $Ba(NO_3)_2$ and two volumes of a saturated soiution of $Ba(OH)_2$.

Filter off the precipitate of phosphates, sulphates, urates, and hippurates and evaporate the filtrate on a water-bath to a thick syrup. This syrup contains chlorides, creatinine, organic salts, pigments, and urea. Extract the syrup with warm 95 per cent alcohol and filter again. The filtrate contains the urea contaminated with pigment. Decolorize the filtrate by boiling with animal charcoal, filter again, and stand the filtrate away in a cold place for crystallization. Examine the crystals under the microscope and compare them with those shown in Fig. 85, page 261.

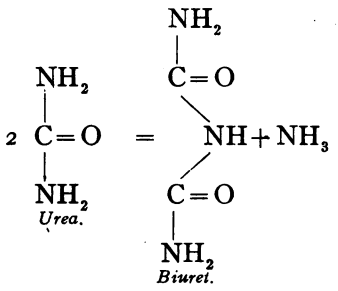
FIG. 87.—MELTING-POINT TUBES FASTENED TO BULB OF THERMOMETER.

2. **Solubility.**—Test the solubility of urea, prepared by yourself or furnished by the instructor, in the ordinary solvents (see p. 22) and in alcohol and ether.

3. **Melting-point.**—Determine the melting-point of some pure urea furnished by the instructor. Proceed as follows: Into an ordinary melting-point tube, sealed at one end, introduce a crystal of urea. Fasten the tube to the bulb of a thermometer as shown in Fig. 87, and suspend the bulb and its attached tube in a small beaker containing sulphuric acid. Gently raise the temperature of the acid by means of a low flame, stirring the fluid continually, and note the temperature at which the urea begins to melt.

4. **Crystalline Form.**—Dissolve a crystal of pure urea in a few drops of 95 per cent alcohol and place 1–2 drops of the alcoholic solution on a microscopic slide. Allow the alcohol to evaporate spontaneously, examine the crystals under the microscope, and compare them with those reproduced in Fig. 85, p. 261. Recrystallize a little urea from water in the same way and compare the crystals with those obtained from the alcoholic solution.

5. **Formation of Biuret.**—Place a small amount of urea in a *dry* test-tube and heat carefully in a low flame. The urea melts at 132° C. and liberates ammonia. Continue heating until the fused mass begins to solidify. Cool the tube, dissolve the residue in dilute potassium hydroxide solution, and add very dilute cupric sulphate solution (see p. 90). The purplish-violet color is due to the presence of biuret which has been formed from the urea through the application of heat as indicated. This is the reaction:



6. **Urea Nitrate.**—Prepare a concentrated solution of urea by dissolving a little of the substance in a few drops of water. Place a drop of this solution on a microscopic slide, add a drop of concentrated nitric acid, and examine under the microscope. Compare the crystals with those reproduced in Fig. 86, p. 263.

7. **Urea Oxalate.**—To a drop of a concentrated solution of urea, prepared as described in the last experiment (6), add a drop of a

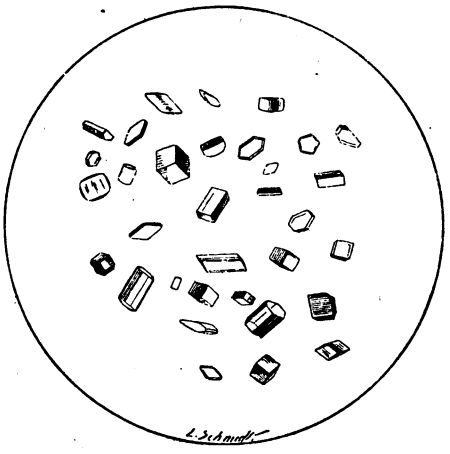


FIG. 88. UREA OXALATE.

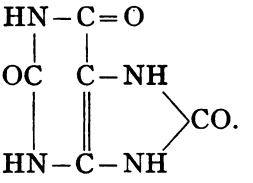
saturated solution of oxalic acid. Examine under the microscope and compare the crystals with those shown in Fig. 88, above.

8. **Decomposition by Sodium Hypobromite.**—Into a mixture of 3 c.c. of concentrated sodium hydroxide solution and 2 c.c. of bromine water in a test-tube introduce a crystal of urea or a small amount of concentrated solution of urea. Through the influence of the sodium hypobromite, NaOBr, the urea is decomposed and carbon dioxide and nitrogen are liberated. The carbon dioxide is absorbed by the excess of sodium hydroxide, while the nitrogen is evolved and causes

the marked effervescence observed. This property forms the basis for one of the methods in common use for the quantitative determination of urea. Write the equation showing the decomposition of urea by sodium hypobromite.

9. **Furfurol Test.**—To a few crystals of urea in a small porcelain dish add 1–2 drops of a concentrated aqueous solution of furfurol and 1–2 drops of a concentrated hydrochloric acid. Note the appearance of a yellow color which gradually changes into a purple. Allantoïn also responds to this test (see page 280).

URIC ACID,



Uric acid is one of the most important of the constituents of the urine. It is generally stated that normally about 0.7 gram is excreted in 24 hours but that this amount is subject to wide variations, particularly under certain dietary and pathological conditions. Very recently it has been shown that the average daily excretion of uric acid for ten men ranging in age from 19 to 29 years and fed a normal mixed diet was 0.597 gram, a value somewhat lower than the generally accepted average of 0.7 gram for such a period. Uric acid is a diureide and consequently upon oxidation yields two molecules of urea. It acts as a weak dibasic acid and forms two classes of salts, neutral and acid. The neutral potassium and lithium urates are the most easily soluble of the alkali salts; the ammonium urate is difficultly soluble. The acid-alkali urates are more insoluble and form the major portion of the sediment which separates upon cooling the concentrated urine; the alkaline earth urates are very insoluble. Ordinarily uric acid occurs in the urine in the form of urates and upon acidifying the liquid the uric acid is liberated and deposits in crystalline form. This property forms the basis of one of the older methods for the quantitative determination of uric acid (Heintz Method, p. 367).

Uric acid is very closely related to the purine bases as may be seen from a comparison of its structural formula with those of the purine bases given on page 237. According to the purine nomenclature it is designated 2–6–8–trioxypurine. Uric acid forms the principal end-product of the nitrogenous metabolism of birds and scaly amphibians; in the human organism it occupies the fourth position inasmuch as here urea, ammonia, and creatinine are the chief end-products of nitrogenous

PLATE V.

URIC ACID CRYSTALS. NORMAL COLOR. (From *Purdy*, after *Peyer*.)

metabolism. It is generally said that the relation existing between uric acid and urea in human urine under normal conditions varies on the average from 1:40 to 1:100 and is subject to wider variations under pathological conditions; and further that because of the high content of uric acid in the urine of new-born infants the ratio may be reduced to 1:10 or even lower. We now know that this ratio of uric acid to urea is of little significance under any conditions.

In man, uric acid probably results principally from the destruction of nuclein material. It may arise from nuclein or other purine material ingested as food or from the disintegrating cellular matter of the organism. The uric acid resulting from the first process is said to be of *exogenous* origin, whereas the product of the second form of activity is said to be of *endogenous* origin. As a result of experimentation, Sivén, and Burian and Schur, and Rockwood claim that the amount of endogenous uric acid formed in any given period is fairly constant for each individual under normal conditions, and that it is entirely independent of the total amount of nitrogen eliminated. Recently Folin has taken exception to the statements of these investigators and claims that, following a pronounced decrease in the amount of protein metabolized, the absolute quantity of uric acid is decreased but that this decrease is relatively smaller than the decrease in the total nitrogen excretion and that the per cent of the uric acid nitrogen, in terms of the total nitrogen, is therefore decidedly increased.

In birds and scaly amphibians the formation of uric acid is analogous to the formation of urea in man. In these organisms it is derived principally from the protein material of the tissues and the food and is formed through a process of synthesis which occurs for the most part in the liver; a comparatively small fraction of the total uric acid excretion of birds and scaly amphibians may result from nuclein material.

When pure, uric acid may be obtained as a white, odorless, and tasteless powder, which is composed principally of small, transparent, crystalline, rhombic plates. Uric acid as it separates from the urine is invariably pigmented, and crystallizes in a large variety of characteristic forms, *e. g.*, dumb-bells, wedges, rhombic prisms, irregular rectangular or hexagonal plates, whetstones, prismatic rosettes, etc. Uric acid is insoluble in alcohol and ether, soluble with difficulty in boiling water (1:1800) and practically insoluble in cold water (1:39,480, at 18° C.). It is soluble in alkalis, alkali carbonates, boiling glycerol, concentrated sulphuric acid, and in certain organic bases such as ethylamine and piperidine. It is claimed that the uric acid is held in solution in the urine by the urea and disodium hydrogen phosphate present.

Uric acid possesses the power of reducing cupric hydroxide in alkaline solution and may thus lead to an erroneous conclusion in testing for sugar in the urine by means of Fehling's or Trommer's tests. A white precipitate of cuprous urate is formed if only a small amount of cupric hydroxide is present, but if enough of the copper salt is present the characteristic red or brownish-red precipitate of cuprous oxide is obtained. Uric acid does not possess the power of reducing bismuth in alkaline solution and therefore does not interfere in testing for sugar in the urine by means of Boettger's or Nylander's tests.

In addition to being an important urinary constituent uric acid is normally present in the brain, heart, liver, lungs, pancreas, and spleen; it also occurs in the blood of birds and has been detected in traces in human blood under normal conditions.

Pathologically, the excretion of uric acid is subject to wide variations, but the experimental findings are rather contradictory. It may be stated with certainty, however, that in leukæmia the uric acid output is increased absolutely as well as relatively to the urea output; under these conditions the ratio between the uric acid and urea may be as low as 1:9, whereas the normal ratio, as we have seen, is 1:50 or higher. In the study of the influence of X-ray on metabolism Edsall has very recently reached some interesting conclusions. He found that the excretion of uric acid is usually increased and that in some conditions, particularly in leukæmia, it may be *greatly* increased. The excretion of total nitrogen, phosphates, and other substances may also be considerably increased.

Experiments on Uric Acid.

1. **Isolation from the Urine.**—Place about 200 c.c. of filtered urine in a beaker, render it acid with 2–10 c.c. of concentrated hydrochloric acid, stir thoroughly, and stand the vessel in a cold place for 24 hours. Examine the pigmented crystals of uric acid under the microscope and compare them with those shown in Fig. 101, p. 342 and Pl. V., opposite p. 267.

2. **Solubility.**—Try the solubility of pure uric acid, furnished by the instructor, in the ordinary solvents (see p. 22) and in alcohol, ether, concentrated sulphuric acid and in boiling glycerol.

3. **Crystalline Form of Pure Uric Acid.**—Place about 100 c.c. of water in a small beaker, render it distinctly alkaline with potassium hydroxide solution and add a small amount of pure uric acid, stirring continuously. Cool the solution, render it distinctly acid with hydro-

chloric acid and allow it to stand in a cool place for crystallization. Examine the crystals under the microscope and compare them with those reproduced in Fig. 89, below.

4. **Murexide Test.**—To a small amount of pure uric acid in a small evaporating dish add 2–3 drops of concentrated nitric acid. Evaporate to dryness carefully on a water-bath or over a very low flame. A red or yellow residue remains which turns purplish-red after cooling the dish and adding a drop of very dilute ammonium hydroxide. The color is due to the formation of *murexide*. If potas-

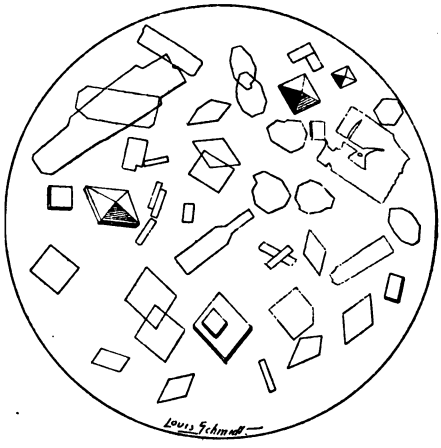


FIG. 89.—PURE URIC ACID.

sium hydroxide is used instead of ammonium hydroxide a purplish-violet color due to the production of the potassium salt is obtained. The color disappears upon warming; with certain related bodies (purine bases) the color persists under these conditions.

5. **Moreigne's Reaction.**—To equal volumes of Moreigne's reagent[1] and the solution to be tested add a few drops of concentrated potassium hydroxide. A blue color indicates the presence of uric acid.

6. **Schiff's Reaction.**—Dissolve a small amount of pure uric acid in sodium carbonate solution and transfer a drop of the resulting mixture to a strip of filter paper saturated with argentic nitrate solution. A yellowish-brown or black coloration due to the formation of reduced silver is produced.

7. **Ganassini's Test.**[2]—Dissolve a small amount of uric acid in

[1] Moreigne's reagent is made by combining 20 grams of sodium tungstate, 10 grams of phosphoric acid (sp. gr. 1.13) and 100 c.c. of water. Boil this mixture for twenty minutes, add water to make the volume of the solution equivalent to the original volume, and acidify with hydrochloric acid.

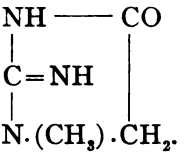
[2] Ganassini: *Boll. soc.*, 1908, No. 1.

sodium carbonate. Precipitate the dissolved uric acid by means of zinc chloride, filter off the precipitate, and permit it to stand in contact with the air. A sky-blue color will develop, a color change which may be hastened by sunlight. A similar reaction may be obtained by treating the original precipitate with $K_2S_2O_8$.

8. **Influence upon Fehling's Solution.**—Dilute 1 c.c. of Fehling's solution with 4 c.c. of water and heat to boiling. Now add *slowly*, a few drops at a time, 1–2 c.c. of a concentrated solution of uric acid in potassium hydroxide, heating after each addition. From this experiment what do you conclude regarding the possibility of arriving at an erroneous decision when testing for sugar in the urine by means of Fehling's test?

9. **Reduction of Nylander's Reagent.**—To 5 c.c. of a solution of uric acid in potassium hydroxide add about one-half a cubic centimeter of Nylander's reagent and heat to boiling for a few moments. Do you obtain the typical black end-reaction signifying the reduction of the bismuth?

CREATININE,



Creatinine is the anhydride of creatine and is a constituent of normal human urine. The theory that creatinine is derived from the creatine of ingested muscular tissue as well as from the creatine of the muscular tissue of the organism has recently been proven to be incorrect by Folin, Klercker, and Wolf and Shaffer. Shaffer believes that creatinine is the result of some special process of normal metabolism which takes place to a large extent, if not entirely, in the muscles and further that the amount of such creatinine elimination, expressed *in milligrams per kilogram body weight*, is an index of this special process.[1] He further states that the muscular efficiency of the individual depends upon the intensity of this process. Under normal conditions about 1 gram of creatinine is excreted by an adult man in 24 hours,[2] the exact amount depending in great part upon the nature of the food and decreasing markedly in starvation. Very little that is important is known regarding the excretion of creatinine under pathological conditions. The creatinine content of the urine is said to be increased in typhoid fever,

[1] He proposes to designate as the "creatinine coefficient" the excretion of *creatinine-nitrogen (mgs.) per kilogram of body weight.*

[2] According to Shaffer the amount excreted by strictly normal individuals is between 7 and 11 milligrams of creatinine-nitrogen per kilogram of body weight.

typhus, tetanus, and pneumonia, and to be decreased in anæmia, chlorosis, paralysis, muscular atrophy, advanced degeneration of the kidneys, and in leucæmia (myelogenous, lymphatic and pseudo). An increase of creatinine was also noted in diabetes, an increase probably due to the creatinine content of the meat eaten. The greater part of the data, however, relating to the variation of the creatinine excretion under pathological conditions are not of much value since in nearly every instance the diet was not sufficiently controlled to permit the collection of reliable data. And further, until the advent of the Folin method (see p. 385), there was no accurate method for the quantitative

FIG. 90.—CREATININE.

determination of creatinine. Shaffer has very recently called attention to the fact that a low excretion of creatinine is found in the urine of a remarkably large number of pathological subjects, representing a variety of conditions, and that it is therefore evident that the excretion of an abnormally small amount of this substance is by no means peculiar to any one disease.

Creatinine crystallizes in colorless, glistening monoclinic prisms (Fig. 90, above) which are soluble in about 12 parts of cold water; they are more soluble in warm water and in warm alcohol. One of the most important and interesting of the compounds of creatinine is *creatinine-zinc chloride*, $(C_4H_7N_3O)_2ZnCl_2$, which is formed from an alcoholic solution of creatinine upon treatment with zinc chloride in acid solution. Creatinine has the power of reducing cupric hydroxide in alkaline solution and in this way may interfere with the determination of sugar in the urine. In the reduction by creatinine the blue liquid is

first changed to a yellow and the formation of a brownish-red precipitate of cuprous oxide is brought about only after continuous boiling with an excess of the copper salt. Creatinine does not reduce alkaline bismuth solutions and therefore does not interfere with Nylander's and Boettger's tests.

It has recently been shown by Folin that the absolute quantity of creatinine eliminated in the urine on a meat-free diet is a constant quantity different for different individuals, but wholly independent of quantitative changes in the total amount of nitrogen eliminated. Shaffer has very recently confirmed these findings and has shown that the output of creatinine under these conditions is constant from hour to hour as well as from day to day.

Experiments on Creatinine.

1. **Separation from the Urine.**—Place 250 c.c. of urine in a casserole or beaker, render it alkaline with milk of lime and then add $CaCl_2$ solution until the phosphates are completely precipitated. Filter off the precipitate, render the filtrate slightly acid with acetic acid, and evaporate it to a syrup. While still warm this syrup is

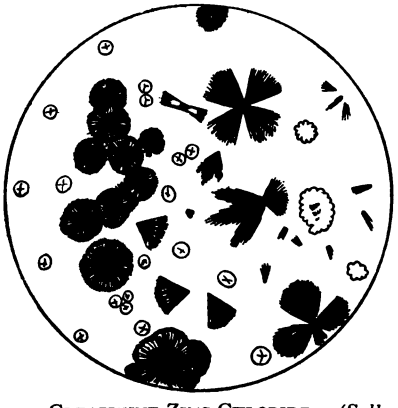


Fig. 91.—Creatinine-Zinc Chloride. (*Salkowski.*)

treated with about 50 c.c. of 95–97 per cent alcohol and the mixture allowed to stand 8–12 hours in a cool place. The precipitate is now filtered off and the filtrate treated with a little sodium acetate and about one-half c.c. of acid-free zinc chloride solution having a specific gravity of 1.2. This mixture is stirred thoroughly and allowed to stand in a cold place for 48–72 hours. Creatinine-zinc chloride (Fig. 91, above) will crystallize out under these conditions. Collect the

crystals on a filter paper and wash them with alcohol to remove chlorides. Now treat the zinc chloride compound with a little warm water, boil with lead oxide and filter. The filtrate may now be decolorized by animal charcoal, evaporated to dryness, and the residue extracted with strong alcohol. (Creatine remains undissolved under these conditions.) The alcoholic extract of creatinine is now evaporated to incipient crystallization and left in a cool place until crystallization is complete. If desired the crystals may be purified by recrystallization from water.

2. **Weyl's Test.**—Take 5 c.c. of urine in a test-tube, add a few drops of sodium nitro-prusside and render the solution alkaline with potassium hydroxide solution. A ruby-red color results which soon turns yellow. See Legal's test for acetone, page 323.

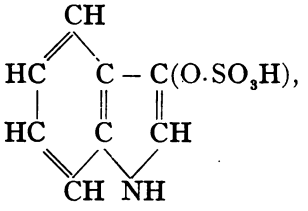
3. **Salkowski's Test.**—To the yellow solution obtained in Weyl's test above add an excess of acetic acid and apply heat. A green color results and is in turn displaced by a blue color. A precipitate of Prussian blue may form.

4. **Jaffe's Reaction.**—Place 5 c.c. of urine in a test-tube, add an aqueous solution of picric acid and render the mixture alkaline with potassium hydroxide solution. A red color is produced which turns yellow if the solution be acidified. Dextrose gives a similar red color but only upon the application of heat. This color reaction observed when creatinine in alkaline solution is treated with picric acid is the basic principle of Folin's colorimetric method for the quantitative determination of creatinine (see page 385).

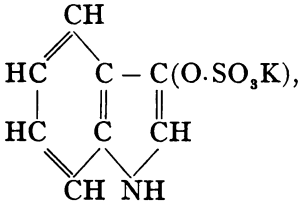
ETHEREAL SULPHURIC ACIDS.

The most important of the ethereal sulphuric acids found in the urine are *phenol-sulphuric acid*, *p-cresol-sulphuric acid*, *indoxyl-sulphuric acid*, and *skatoxyl-sulphuric acid*. Pyrocatechin-sulphuric acid also occurs in traces in human urine. The total output of ethereal sulphuric acid varies from 0.09 to 0.62 gram for 24 hours. In health the ratio of ethereal sulphuric acid to inorganic sulphuric acid is about 1 : 10. These ethereal sulphuric acids originate in part from the phenol, cresol, indole and skatole formed in the putrefaction of protein material in the intestine. The phenol passes to the liver where it is conjugated to form phenol potassium sulphate and appears in this form in the urine whereas the indole and skatole undergo a preliminary oxidation to form *indoxyl* and *skatoxyl* respectively before their elimination.

It has generally been considered that each of the ethereal sulphuric acids was formed principally in the putrefaction of protein material in the intestine and that therefore a determination of the total ethereal sulphuric acid content of the urine was an index of the extent to which these putrefactive processes were proceeding within the organism. Recently, however, Folin has conducted a series of experiments which seem to show that the ethereal sulphuric acid content of the urine does *not* afford an index of the extent of intestinal putrefaction, since these bodies arise only in part from putrefactive processes. He claims that the ethereal sulphuric acid excretion represents a form of sulphur metabolism which is more in evidence upon a diet containing a very small amount of protein or upon a diet containing absolutely no protein. The ethereal sulphuric acid content of the urine diminishes as the total sulphur content diminishes but the *percentage decrease* is much less. Therefore when considered from the standpoint of the total sulphuric acid content the ethereal sulphuric acid content is not diminished but is *increased*, although the total sulphuric acid content *is diminished.* Folin's experiments also seem to show that the indoxyl sulphuric acid (indoxyl potassium sulphate or indican) content of the urine does not originate to any degree from the metabolism of protein material but that it arises in great part from intestinal putrefaction and that the excretion of indoxyl sulphuric acid may *alone* be taken as a rough index of the extent of putrefactive processes within the intestine. Indoxyl sulphuric acid,



therefore, which occurs in the urine as indoxyl potassium sulphate or indican,

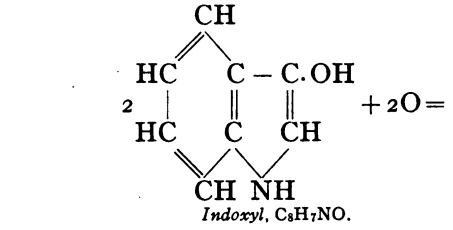


is clinically the most important of the ethereal sulphuric acids.

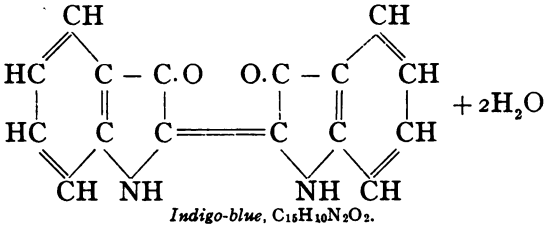
Tests for Indican.

1. **Jaffe's Test.**—Nearly fill a test-tube with a mixture composed of equal volumes of concentrated HCl and the urine under examination. Add 2–3 c.c. of chloroform and a few drops of a calcium hypochlorite solution, place the thumb over the end of the test-tube and shake the tube and contents thoroughly. The chloroform is colored more or less, according to the amount of indican present. Ordinarily a blue color due to the formation of indigo-blue is produced; less frequently a red color due to indigo-red may be noted.

This is the reaction (see also pages 158 and 159):



Indoxyl, C_8H_7NO.



Indigo-blue, $C_{16}H_{10}N_2O_2$.

2. **Obermayer's Test.**—Nearly fill a test-tube with a mixture composed of equal volumes of Obermayer's reagent[1] and the urine under examination. Add 2–3 c.c. of chloroform, place the thumb over the end of the test-tube and shake thoroughly. How does this compare with Jaffe's test?

3. **Gürber's Reaction.**—To one volume of the urine under examination and two volumes of concentrated hydrochloric acid in a test-tube add 2–3 drops of a 1 per cent solution of osmic acid and 2–3 c.c. of chloroform and shake the tube and contents thoroughly. Compare the color with those obtained in Jaffe's and Obermayer's tests.

An excess of osmic acid does not affect the reaction. Occasionally better results are obtained if the solution of osmic acid is added directly to the urine *before* the addition of the hydrochloric acid. If the urine under examination be strongly colored or of high specific gravity it should first be treated with basic lead acetate (one-eighth volume).

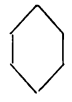
[1] Obermayer's reagent is prepared by adding 2–4 grams of ferric chloride to a liter of concentrated HCl (sp. gr. 1.19).

The precipitate is then removed by filtration and the resulting filtrate used in making the test for indican.

4. **Rossi's Reaction.**—To equal volumes of concentrated hydrochloric acid and the urine under examination, in a test-tube, add 1 drop of a 10 per cent solution of ammonium persulphate and 2–3 c.c. of chloroform. Agitate the mixture vigorously and note the color of the chloroform. Compare this result with those obtained in the other indican tests.

5. **Lavelle's Reaction.**—To 10 c.c. of urine in a test-tube add 2–3 c.c. of Obermayer's reagent[1] and a similar volume of concentrated sulphuric acid. (During the addition of the acid the tube should be held under running water in order that the temperature of the mixture may not rise too high.) Add 2–3 c.c. of chloroform, shake the tube vigorously, and observe the depth of color assumed by the chloroform.

The sponsor for this reaction claims it to be the most satisfactory of the indican tests.

HIPPURIC ACID,  $CO.NH.CH_2.COOH.$

This acid occurs normally in the urine of both the carnivora and herbivora but is more abundant in the urine of the latter. It is formed

FIG. 92.—HIPPURIC ACID.

by a synthesis of benzoic acid and glycocoll which takes place in the kidneys. The average excretion of an adult man for 24 hours under

[1] Obermayer's reagent is prepared by adding 2–4 grams of ferric chloride to a liter of concentrated HCl (sp. gr. 1.19).

normal conditions is about 0.7 gram. Hippuric acid crystallizes in needles or rhombic prisms (see Fig. 92, p. 276) the particular form depending upon the rapidity of crystallization. Pure hippuric acid melts at 187° C. The most satisfactory method for the isolation of hippuric acid from the urine in crystalline form is that proposed by Roaf (see below). It is easily soluble in alcohol or hot water, and only slightly soluble in ether. The output of hippuric acid is increased in diabetes owing probably to the ingestion of much protein and fruit. It is decreased in fevers and in certain kidney disorders where the synthetic activity of the renal cells is diminished. Hippuric acid may be determined quantitatively by means of Dakin's methods (see p. 376).

Experiments on Hippuric Acid.

1. **Separation from the Urine.** (*a*) *First Method.*—Render 500–1000 c.c. of urine of the horse or cow[1] alkaline with milk of lime, boil for a few moments and filter while hot. Concentrate the filtrate, over a burner, to a small volume. Cool the solution, acidify it strongly with concentrated hydrochloric acid and stand it in a cool place for 24 hours. Filter off the crystals of hippuric acid which have formed and wash them with a little cold water. Remove the crystals from the paper, dissolve them in a very small amount of hot water and percolate the hot solution through thoroughly washed animal charcoal, being careful to wash out the last portion of the hippuric acid solution with hot water. Filter, concentrate the filtrate to a small volume and stand it aside for crystallization. Examine the crystals under the microscope and compare them with those in Fig. 92, page 276. This method is not as satisfactory as Roaf's method (see below).

(*b*) *Roaf's Method.*—Place 500 c.c. of urine of the horse or cow[1] in a casserole or precipitating jar and add an equal volume of a saturated solution of ammonium sulphate[2] and 7.5 c.c. of concentrated sulphuric acid. Permit the mixture to stand for twenty-four hours and remove the crystals of hippuric acid by filtration. Purify the crystals by recrystallization according to the directions given above under First Method. Examine the crystals under the microscope and compare them with those given in Fig. 92 p. 276.

[1] If urine of the horse or cow is not available human urine may serve the purpose fully as well provided means are taken to increase its content of hippuric acid. This may be conveniently accomplished by ingesting 2 grams of ammonium benzoate at night. The fraction of urine passed in the morning will be found to have a high content of hippuric acid. The ammonium benzoate is in no way harmful.

[2] 125 grams of *solid* ammonium sulphate may be substituted.

If sufficient urine is not available to permit the use of 500 c.c. a smaller volume may be used inasmuch as it is possible, by the above technique, to isolate hippuric acid in crystalline form from as small a volume as 25–50 c.c. of herbivorous urine. The greater the amount of ammonium sulphate added the more rapid the crystallization until at the saturation point the crystals of hippuric acid sometimes form in about *ten minutes*.

2. **Melting-point.**—Determine the melting-point of the hippuric acid prepared in the above experiment (see p. 264).

3. **Solubility.**—Test the solubility of hippuric acid in the ordinary solvents (page 22) and in alcohol, and ether.

4. **Dehn's Reaction.**—Introduce about 5 c.c. of the urine or the solution under examination into a test-tube and add sufficient hypobromite solution[1] to impart to the mixture a permanent yellow color. In the case of urine enough hypobromite should be added to decompose the urea. Heat the mixture to boiling and note the formation of an orange or brown-red precipitate if hippuric acid is present. If the solution under examination contains only a trace of hippuric acid the solution will appear smoky and faintly red in color, whereas if a larger amount of the acid be present the solution will become opaque and of an orange or brown-red color. In either case after standing for some time the solution should clear up and a light, finely divided precipitate should be deposited. This precipitate consists of earthy phosphates mixed with an amorphous orange or brown-red substance of unknown composition.

5. **Formation of Nitro-Benzene.**—To a little hippuric acid in a small porcelain dish add 1–2 c.c. of concentrated HNO_3 and evaporate to dryness on a water-bath. Transfer the residue to a dry test-tube, apply heat, and note the odor of the artificial oil of bitter almonds (nitro-benzene).

6. **Sublimation.**—Place a few crystals of hippuric acid in a dry test-tube and apply heat. The crystals are reduced to an oily fluid which solidifies in a crystalline mass upon cooling. When stronger heat is applied the liquid assumes a red color and finally yields a sublimate of benzoic acid and the odor of hydrocyanic acid.

7. **Formation of Ferric Salt.**—Render a small amount of a solution of hippuric acid neutral with dilute potassium hydroxide. Now add 1–3 drops of neutral ferric chloride solution and note the formation of the ferric salt of hippuric acid as a cream colored precipitate.

[1] See note on p. 369.

OXALIC ACID, $\begin{matrix} COOH \\ | \\ COOH. \end{matrix}$

Oxalic acid is a constituent of normal urine, about 0.02 gram being eliminated in 24 hours. It is present in the urine as calcium oxalate, which is kept in solution through the medium of the acid phosphates. The origin of the oxalic acid content of the urine is not well understood. It is eliminated, at least in part, unchanged when ingested, therefore since many of the common articles of diet, *e. g.*, asparagus, apples, cabbage, grapes, lettuce, spinach, tomatoes, etc., contain oxalic acid it seems probable that the ingested food supplies a portion of the oxalic acid found in the urine. There is also experimental evidence that part of the oxalic acid of the urine is formed within the organism in the course of protein and fat metabolism. It has also been suggested that oxalic acid may arise from an incomplete combustion of carbohydrates, especially under certain abnormal conditions. Pathologically, oxalic acid is found to be increased in amount in diabetes mellitus, in organic diseases of the liver, and in various other conditions which are accompanied by a derangement of the oxidation mechanism. An abnormal increase of oxalic acid is termed *oxaluria*. A considerable increase in the content of oxalic acid may be noted unaccompanied by any other apparent symptom. Calcium oxalate crystallizes in at least two distinct forms, *dumb-bells* and *octahedra* (Fig. 99, page 340).

Experiments.

Preparation of Calcium Oxalate.—*First Method.*—Place 200–250 c.c. of urine in a beaker, add 5 c.c. of a saturated solution of calcium chloride, make the urine slightly acid with acetic acid, and stand the beaker aside in a cool place for 24 hours. Examine the sediment under the microscope and compare the crystalline forms with those shown in Fig. 99, p. 340.

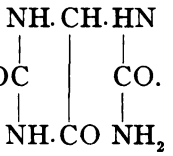
Second Method.—Proceed as above, replacing the acetic acid by an excess of ammonium hydroxide and filtering off the precipitate of phosphates.

NEUTRAL SULPHUR COMPOUNDS.

Under this head may be classed such bodies as cystine (see p. 70), chondroitin-sulphuric acid, oxyproteic acid, alloxyproteic acid, uroferric acid, thiocyanates, and taurine derivatives. The sulphur

content of the bodies just enumerated is generally termed loosely combined or neutral sulphur in order that it may not be confused with the acid sulphur which occurs in the inorganic sulphuric acid and ethereal sulphuric acid forms. Ordinarily the neutral sulphur content of normal human urine is 14–20 per cent of the total sulphur content.

ALLANTOIN,



Allantoïn has been found in the urine of suckling calves as well as in that of the dog and cat. It has also been detected in the urine of infants within the first eight days after birth, as well as in the urine of adults. It is more abundant in the urine of women during pregnancy. Underhill also reports the presence of allantoïn in the urine

FIG. 93.—ALLANTOIN, FROM CAT'S URINE.

a and *b*, Forms in which it crystallized from the urine; *c*, recrystallized allantoïn. (Drawn from micro-photographs furnished by Prof. Lafayette B. Mendel of Yale University.)

of fasting dogs, an observation which makes it probable that allantoïn is a constant constituent of the urine of such animals. Allantoïn is formed by the oxidation of uric acid and the output is increased by thymus or pancreas feeding. When pure it crystallizes in prisms (Fig. 93, above) and when impure in granules and knobs. Pathologically, it has been found increased in diabetes insipidus and in hysteria with convulsions (Pouchet). Mendel and Dakin[1] have recently shown that allantoïn is optically inactive notwithstanding

[1] Mendel and Dakin: *Jour. Biol. Chem.*, VII, p. 153, 1910.

the fact that it contains an asymmetric carbon atom. This phenomenon they believe to be due to tautomeric change.

Experiments.

1. **Separation from the Urine.**[1]—*Meissner's Method.*—Precipitate the urine with baryta water. Neutralize the filtrate *carefully* with dilute sulphuric acid, filter immediately, and evaporate the filtrate to incipient crystallization. Completely precipitate this *warm* fluid with 95 per cent alcohol (reserve the precipitate). Decant or filter and precipitate the solution by ether. Combine the ether and alcohol precipitates and extract with *cold* water or *hot* alcohol; allantoïn remains undissolved. Bring the allantoïn into solution in *hot* water and recrystallize.

Allantoïn may be determined quantitatively by the Paduschka-Underhill-Kleiner method (see p. 401) or by Loewi's method.[2]

2. **Preparation from Uric Acid.**—Dissolve 4 grams of uric acid in 100 c.c. of water rendered alkaline with potassium hydroxide. Cool and *carefully* add 3 grams of potassium permanganate. Filter, *immediately* acidulate the filtrate with acetic acid and allow it to stand in a cool place over night. Filter off the crystals and wash them with water. Save the wash water and filtrate, unite them and after concentrating to a small volume stand away for crystallization. Now combine all the crystals and recrystallize them from hot water. Use these crystals in the experiments which follow.

3. **Microscopical Examination.**—Examine the crystals made in the last experiment and compare them with those shown in Fig. 93, page 280.

4. **Solubility.**—Test the solubility of allantoïn in the ordinary solvents (page 22.)

5. **Reaction.**—Dissolve a crystal in water and test the reaction to litmus.

6. **Furfurol Test.**—Place a few crystals of allantoïn on a test-tablet or in a porcelain dish and add 1–2 drops of a concentrated aqueous solution of furfurol and 1–2 drops of concentrated hydrochloric acid. Observe the formation of a yellow color which turns to a light purple if allowed to stand. This test is given by urea but not by uric acid.

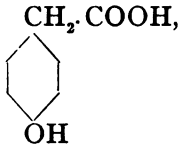
7. **Murexide Test.**—Try this test according to the directions given on page 269. Note that allantoïn fails to respond.

[1] The urine of the dog after thymus, pancreas, or uric acid feeding may be employed.
[2] Archiv für Experimentelle Pathologie und Pharmakologie, 1900, XLIV, p. 20.

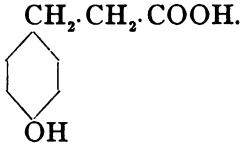
8. **Reduction of Fehling's Solution.**—Make this test in the usual way (see p. 27) except that the boiling must be prolonged and excessive. Ultimately the allantoïn will reduce the solution. Compare with the result on uric acid, page 270.

AROMATIC OXYACIDS.

Two of the most important of the oxyacids are *paraoxyphenyl-acetic acid*,

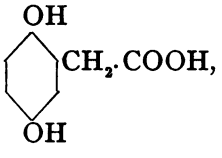


and *paraoxyphenyl-propionic acid*,



They are products of the putrefaction of protein material and tyrosine is an intermediate stage in their formation. Both these acids for the most part pass unchanged into the urine where they occur normally in very small amount. The content may be increased in the same manner as the phenol content, in particular by acute phosphorous poisoning. A fraction of the total aromatic oxyacid content of the urine is in combination with sulphuric acid, but the greater part is present in the form of salts of sodium and potassium.

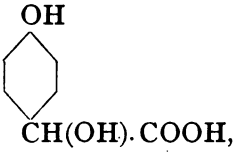
Homogentisic Acid or di-oxyphenyl-acetic acid,



is another important oxyacid sometimes present in the urine. Under the name *glycosuric acid* it was first isolated from the urine by Prof. John Marshall of the University of Pennsylvania; subsequently Baumann isolated it and determined its chemical constitution. It occurs in cases of *alcaptonuria*. A urine containing this oxyacid turns greenish-brown from the surface downward when treated with a little sodium

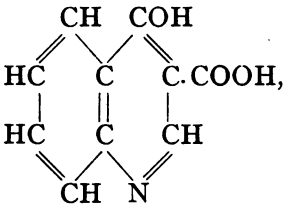
hydroxide or ammonia. If the solution be stirred the color very soon becomes dark brown or even black. Homogentisic acid reduces alkaline copper solutions but not alkaline bismuth solutions. Uroleucic acid is similar in its reactions to homogentisic acid.

Oxymandelic Acid or paraoxyphenyl-glycolic acid,



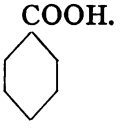
has been detected in the urine in cases of yellow atrophy of the liver.

Kynurenic Acid or γ-oxy-β-quinoline carbonic acid,



is present in the urine of the dog and has recently been detected by Swain in the urine of the coyote. To isolate it from the urine proceed as follows: Acidify the urine with hydrochloric acid in the proportion 1 : 25. From this acid fluid both the uric acid and the kynurenic acid separate in the course of 24–48 hours. Filter off the combined crystalline deposit of the two acids, dissolve the kynurenic acid in dilute ammonia (uric acid is insoluble), and reprecipitate it with hydrochloric acid.

Kynurenic acid may be quantitatively determined by Capaldi's method.[1]

BENZOIC ACID, 

Benzoic acid has been detected in the urine of the rabbit and dog. It is also said to occur in human urine accompanying renal disorders. The benzoic acid probably originates from a fermentative decomposition of the hippuric acid of the urine.

[1] *Zeitschrift für physiologische Chemie*, 1897, XXIII, p. 92.

EXPERIMENTS.

1. **Solubility.**—Test the solubility of benzoic acid in water, alcohol, and ether.

2. **Crystalline Form.**—Recrystallize some benzoic acid from hot water, examine the crystals under the microscope, and compare them with those reproduced in Fig. 94, below.

3. **Sublimation.**—Place a little benzoic acid in a test-tube and heat over a flame. Note the odor which is evolved and observe that the acid sublimes in the form of needles.

FIG. 94. BENZOIC ACID.

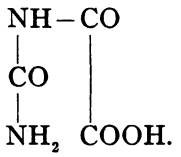
4. Dissolve a little sodium benzoate in water and add a solution of neutral ferric chloride. Note the production of a brownish-yellow precipitate (salicylic acid gives a reddish-violet color under the same conditions). Add ammonium hydroxide to some of the precipitate. It dissolves and ferric hydroxide is formed. Add a little hydrochloric acid to another portion of the original precipitate and stand the vessel away over night. What do you observe?

NUCLEOPROTEIN.

The nubecula of normal urine has been shown by one investigator to consist of a mucoid containing 12.7 per cent of nitrogen and 2.3 per cent of sulphur. This body evidently originates in the urinary passages. It is probably slightly soluble in the urine. Some investigators believe that the body forming the nubecula of normal urine is nucleoprotein and not a mucin or mucoid as stated above.

A discussion of nucleoprotein and related bodies occurring in the urine under pathological conditions will be found on page 315.

OXALURIC ACID,



Oxaluric acid is not a constant constituent of normal human urine, and when found occurs only in traces as the ammonium salt. Upon boiling oxaluric acid it splits into oxalic acid and urea.

ENZYMES.

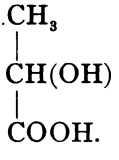
Various types of enzymes produced within the organism are excreted in both the feces and the urine. In this connection it is interesting to note that *pepsin*, *gastric rennin*, and an *amylase* have been positively identified in the urine. The occurrence of trypsin in the urine, at least under normal conditions, is questioned.

VOLATILE FATTY ACIDS.

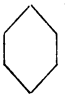
Acetic, butyric, and formic acids have been found under normal conditions in the urine of man and of certain carnivora as well as in the urine of herbivora. Normally they arise principally from the fermentation of carbohydrates and the putrefaction of proteins. The acids containing the fewest carbon atoms (formic and acetic) are found to be present in larger percentage than those which contain a larger number of such atoms. The volatile fatty acids occur in normal urine in traces, the total output for twenty-four hours, according to different investigators, varying from 0.008 gram to 0.05 gram.

Pathologically, the excretion of volatile fatty acids is increased in diabetes, fevers, and in certain hepatic diseases in which the parenchyma of the liver is seriously affected. Under other pathological conditions the output may be diminished. These variations, however, in the excretion of the volatile fatty acids possess very little diagnostic value.

PARALACTIC ACID,



Paralactic acid is supposed to pass into the urine when the supply of oxygen in the organism is diminished through any cause, *e. g.*, after acute yellow atrophy of the liver, acute phosphorus poisoning, or epileptic attacks. This acid has also been found in the urine of healthy persons following the physical exercise incident to prolonged marching. Paralactic acid has been detected in the urine of birds after the removal of the liver. Underhill reports the occurrence of this acid in the urine of a case of pernicious vomiting of pregnancy.

PHENACETURIC ACID,  $CH_2 \cdot CO \cdot NH \cdot CH_2 \cdot COOH$.

Phenaceturic acid occurs principally in the urine of herbivorous animals but has frequently been detected in human urine. It is produced in the organism through the synthesis of glycocoll and phenylacetic acid. It may be decomposed into its component parts by boiling with dilute mineral acids. The crystalline form of phenaceturic acid (small rhombic plates with rounded angles) resembles one form of uric acid crystal.

PHOSPHORIZED COMPOUNDS.

Phosphorus in organic combination has been found in the urine in such bodies as glycerophosphoric acid, which may arise from the decomposition of lecithin, and phosphocarnic acid. It is claimed that on the average about 2.5 per cent of the total phosphorus elimination is in organic combination.

PIGMENTS.

There are at least three pigments normally present in human urine. These pigments are *urochrome*, *urobilin*, and *uroerythrin*.

A. UROCHROME.

This is the principal pigment of normal urine and imparts the characteristic yellow color to that fluid. It is apparently closely related to its associated pigment urobilin since the latter may be readily converted into urochrome through evaporation of its aqueous-ether solution. Urochrome may be obtained in the form of a brown, amorphous powder which is readily soluble in water and 95 per cent alcohol. It is less soluble in absolute alcohol, acetone, amyl alcohol, and acetic

ether and insoluble in benzene, chloroform, and ether. Urochrome is said to be a nitrogenous body (4.2 per cent nitrogen), free from iron.

B. UROBILIN.

Urobilin, which was at one time considered to be the principal pigment of urine, in reality contributes little toward the pigmentation of this fluid. It is claimed that no urobilin is present in freshly voided normal urine but that its precursor, a chromogen called *urobilinogen*, is present and gives rise to urobilin upon decomposition through the influence of light. It is claimed by some investigators that there are various forms of urobilin, *e. g.*, normal, febrile, physiological, and pathological. Urobilin is said to be very similar to, if not absolutely identical with, hydrobilirubin (see page 169).

Urobilin may be obtained as an amorphous powder which varies in color from brown to reddish-brown, red and reddish-yellow, depending upon the way in which it is prepared. It is easily soluble in ethyl alcohol, amyl alcohol, and chloroform, and slightly soluble in ether, acetic ether, and in water. Its solutions show characteristic absorption-bands (see Absorption Spectra, Plate II). Under normal conditions urobilin is derived from the bile pigments in the intestine.

Urobilin is increased in most acute infectious diseases such as *erysipelas*, *malaria*, *pneumonia*, and *scarlet fever*. It is also increased in *appendicitis*, *carcinoma of the liver*, *catarrhal icterus*, *pernicious anæmia*, and in cases of poisoning by antifebrin, antipyrin, pyridin, and potassium chlorate. In general it is usually increased when blood destruction is excessive and in many disturbances of the liver. It is markedly decreased in phosphorus poisoning.

EXPERIMENTS.

1. **Spectroscopic Examination.**—Acidify the urine with hydrochloric acid and allow it to remain exposed to the air for a few moments. By this means if any urobilinogen is present it will be transformed into urobilin. The urine may now be examined by means of the spectroscope. If urobilin is present in the fluid the characteristic absorption-band lying between *b* and *F* will be observed (see Absorption Spectra, Plate II). It may be found necessary to dilute the urine with water before a distinct absorption-band is observed. This test may be modified by acidifying 10 c.c. of urine with hydrochloric acid and shaking it gently with 5 c.c. of amyl alcohol. The alcoholic extract when examined spectroscopically will show the characteristic

urobilin absorption-band. (Note the spectroscopic examination in the next experiment.)

2. **Ammoniacal-zinc Chloride Test.**—Render some of the urine ammoniacal by the addition of ammonium hydroxide, and after allowing it to stand a short time filter off the precipitate of phosphates and add a few drops of zinc chloride solution to the filtrate. Observe the production of a greenish fluorescence. Examine the fluid by means of the spectroscope and note the absorption-band which occupies much the same position as the absorption-band of urobilin in acid solution (see Absorption Spectra, Plate II).

3. **Gerhardt's Test.**—To 20 c.c. of urine add 3–5 c.c. of chloroform and shake well. Separate the chloroform extract and add to it a few drops of iodine solution (I in KI). Render the mixture alkaline with dilute solution of potassium hydroxide and note the production of a yellow or yellowish-brown color. The solution ordinarily exhibits a greenish fluorescence.

4. **Wirsing's Test.**—To 20 c.c. of urine add 3–5 c.c. of chloroform and shake gently. Separate the chloroform extract and add to it a drop of an alcoholic solution of zinc chloride. Note the rose-red color and the greenish fluorescence. If the solution is turbid it may be rendered clear by the addition of a few c.c. of absolute alcohol.

5. **Ether-Absolute Alcohol Test.**—Mix urine and pure ether in equal volumes and shake gently in a separatory funnel. Separate the ether extract, evaporate it to dryness, and dissolve the residue in 2–3 c.c. of absolute alcohol. Note the greenish fluorescence. Examine the solution spectroscopically and observe the characteristic absorption-band (see Absorption Spectra, Plate II).

6. **Ring Test.**—Acidify 25 c.c. of urine with 2–3 drops of concentrated hydrochloric acid, add 5 c.c. of chloroform and shake the mixture. Separate the chloroform, place it in a test-tube, and add carefully 3–5 c.c. of an alcoholic solution of zinc acetate. Observe the formation of a green ring at the zone of contact of the two fluids. If the tube is shaken a fluorescence may be observed.

C. UROERYTHRIN.

This pigment is frequently present in small amount in normal urine. The red color of urinary sediments is due in great part to the presence of uroerythrin. It is easily soluble in amyl alcohol, slightly soluble in acetic ether, absolute alcohol, or chloroform, and nearly insoluble in water. Dilute solutions of uroerythrin are pink in color while concentrated solutions are orange-red or bright red: none of its

solutions fluoresce. Uroerythrin is increased in amount after strenuous physical exercise, digestive disturbances, fevers, certain liver disorders, and in various other pathological conditions.

PTOMAINES AND LEUCOMAINES.

These toxic substances are said to be present in small amount in normal urine. Very little is known, definitely, however, about them. It is claimed that five different poisons may be detected in the urine, and it is further stated that each of these substances produces a specific and definite symptom when injected intravenously into a rabbit. The resulting symptoms are narcosis, salivation, mydriasis, paralysis, and convulsions. The day urine is principally narcotic and is 2–4 times as toxic as the night urine which is chiefly productive of convulsions.

PURINE BASES.

The purine bases found in human urine are adenine, carnine, epiguanine, episarkine, guanine, xanthine, heteroxanthine, hypoxanthine, paraxanthine, and 1-methylxanthine. The main bulk of the purine base content of the urine is made up of *paraxanthine*, *heteroxanthine* and 1-*methylxanthine* which are derived for the most part from the caffeine, theobromine, and theophylline of the food. The total purine base content is made up of the products of two distinct forms of metabolism, *i. e.*, metabolism of ingested nucleins and purines and metabolism of tissue nuclein material. Purine bases resulting from the first form of metabolism are said to be of *exogenous* origin whereas those resulting from the second form of metabolism are said to be of *endogenous* origin. The daily output of purine bases by the urine is extremely small and varies greatly with the individual (16–60 milligrams). The output is increased after the ingestion of nuclein material as well as after the increased destruction of leucocytes. A well marked increase accompanies leukæmia. Edsall has very recently shown that the output of purine bases by the urine is increased as a result of X-ray treatment.

EXPERIMENT.

1. **Formation of the Silver Salts.**—Add an excess of magnesia mixture[1] to 25 c.c. of urine. Filter off the precipitate and add am-

[1] Magnesia mixture may be prepared as follows: Dissolve 175 grams of $MgSO_4$ and 350 grams of NH_4Cl in 1400 c.c. of distilled water. Add 700 grams of concentrated NH_4OH, mix very thoroughly and preserve the mixture in a glass-stoppered bottle.

moniacal silver solution[1] to the filtrate. A precipitate composed of the silver salts of the various purine bases is produced. The purine bases may be determined quantitatively by means of Krüger and Schmidt's method (see p. 399), or Welker's method (see p. 398).

2. Inorganic Physiological Constituents.

Ammonia.

Next to urea, ammonia is the most important of the nitrogenous end-products of protein metabolism. Ordinarily about 2.5–4.5 per cent of the total nitrogen of the urine is eliminated as ammonia and on the average this would be about 0.7 gram per day. Under normal conditions the ammonia is present in the urine in the form of the *chloride*, *phosphate*, or *sulphate*. This is due to the fact that combinations of this sort are not oxidized in the organism to form urea, but are excreted as such. This explains the increase in the output of ammonia which follows the administration of the ammonium salts of the mineral acids or of the acids themselves. On the other hand, when ammonium acetate and many other ammonium salts of certain organic acids are administered no increase in the output of ammonia occurs since the salt is oxidized and its nitrogen ultimately appears in the urine as urea.

The acids formed during the process of protein destruction within the body have an influence upon the excretion of ammonia similar to that exerted by acids which have been administered. Therefore a pathological increase in the output of ammonia is observed in such diseases as are accompanied by an increased and imperfect protein metabolism, and especially in diabetes, in which disease diacetic acid and β-oxybutyric acid are found in the urine in combination with the ammonia.

As the result of recent experiments Folin claims that a pronounced decrease in the extent of protein metabolism, as measured by the total nitrogen in the urine, is frequently accompanied by a decreased elimination of ammonia. The ammonia elimination is therefore probably determined by other factors than the total protein catabolism as such. Furthermore, he believes that a decided decrease in the total nitrogen excretion is always accompanied by a *relative increase* in the ammonia-nitrogen, provided the food is of a character yielding an alkaline ash.

[1] Ammoniacal silver solution may be prepared according to directions given on page 401.

The quantitative determination of ammonia must be made upon the fresh urine since upon standing the normal urine will undergo ammoniacal fermentation (see page 253).

Sulphates.

Sulphur in combination is excreted in two forms in the urine; first, as *loosely combined*, *unoxidized* or *neutral sulphur*, and, second, as *oxidized* or *acid* sulphur. The *loosely combined* sulphur is excreted mainly as a constituent of such bodies as cystine, cysteine, taurine, hydrogen sulphide, ethyl sulphide, thiocyanates, sulphonic acids, oxyproteic acid, alloxyproteic acid, and uroferric acid. The amount of loosely combined sulphur eliminated is in great measure independent of the extent of protein decomposition or of the total sulphur excretion. In this characteristic it is somewhat similar to the excretion of creatinine. The *oxidized* sulphur is eliminated in the form of sulphuric acid, principally as salts of sodium, potassium, calcium, and magnesium; a relatively small amount occurs in the form of *ethereal* sulphuric acid, *i. e.*, sulphuric acid in combination with such *aromatic* bodies as phenol, indole, skatole, cresol, pyrocatechin, and hydroquinone. Sulphuric acid in combination with Na, K, Ca or Mg is sometimes termed *inorganic* or *preformed sulphuric acid*, whereas the ethereal sulphuric acid is sometimes called *conjugate sulphuric acid*. The greater part of the sulphur is eliminated in the oxidized form, but the absolute percentage of sulphur excreted as the preformed, ethereal or loosely combined type depends upon the total quantity of sulphur present, *i. e.*, there is no definite ratio between the three forms of sulphur which will apply under all conditions. The preformed sulphuric acid may be precipitated directly from acidified urine with $BaCl_2$, whereas the ethereal sulphuric acid must undergo a preliminary boiling in the presence of a mineral acid before it can be so precipitated.

The sulphuric acid excreted by the urine arises principally from the oxidation of protein material within the body; a relatively small amount is due to ingested sulphates. Under normal conditions about 2.5 grams of sulphuric acid is eliminated daily. Since the sulphuric acid content of the urine has, for the most part, a protein origin and since one of the most important constituents of the protein molecule is nitrogen, it would be reasonable to suppose that a fairly definite ratio might exist between the excretion of these two elements. However, when we appreciate that the percentage content of N and S present in different proteins is subject to rather wide variations, the

fixing of a ratio which will express the exact relation existing between these two substances, as they appear in the urine as end-products of protein metabolism, is practically impossible. It has been suggested that the ratio 5 : 1 expresses this relation in a general way.

Pathologically, the excretion of sulphuric acid by the urine is increased in acute fevers and in all other diseases marked by a stimulated metabolism, whereas a decrease in the sulphuric acid excretion is observed in those diseases which are accompanied by a loss of appetite and a diminished metabolic activity.

Experiments.

1. **Detection of Inorganic Sulphuric Acid.**—Place about 10 c.c. of urine in a test-tube, acidify with acetic acid and add some barium chloride solution. A white precipitate of barium sulphate forms.

2. **Detection of Ethereal Sulphuric Acid.**—Filter off the barium sulphate precipitate formed in the above experiment, add 1 c.c. of hydrochloric acid and a little barium chloride solution to the filtrate and heat the mixture to boiling for 1–2 minutes. Note the appearance of a turbidity due to the presence of sulphuric acid which has been separated from the ethereal sulphates and has combined with the barium of the $BaCl_2$ to form $BaSO_4$.

3. **Detection of Loosely Combined or Neutral Sulphur.**—Place about 10 c.c. of urine in a test-tube, introduce a small piece of zinc, add sufficient hydrochloric acid to cause a gentle evolution of hydrogen, and over the mouth of the tube place a filter paper saturated with plumbic acetate solution. In a short time the portion of the paper in contact with the vapors within the test-tube becomes blackened due to the formation of lead sulphide. The nascent hydrogen has reacted with the loosely combined or neutral sulphur to form hydrogen sulphide and this gas coming in contact with the plumbic acetate paper has caused the production of the black lead sulphide. Sulphur in the form of inorganic or ethereal sulphuric acid does not respond to this test.

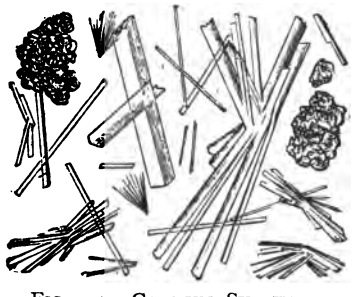


Fig. 95.—Calcium Sulphate. (*Hensel* and *Weil.*)

4. **Calcium Sulphate Crystals.**—Place 10 c.c. of urine in a test-tube, add 10 drops of calcium chloride solution and allow the tube to stand until crystals form. Examine the calcium sulphate crystals

under the microscope and compare them with those shown in Fig. 95, page 292.

Chlorides.

Next to urea, the chlorides constitute the chief *solid* constituent of the urine. The principal chlorides found in the urine are those of sodium, potassium, ammonium, and magnesium, with sodium chloride predominating. The excretion of chloride is dependent, in great part, upon the nature of the diet, but on the average the daily output is about 10–15 grams, expressed as sodium chloride. Copious water-drinking increases the output of chlorides considerably. Because of their solubility, chlorides are never found in the urinary sediment.

Since the amount of chlorides excreted in the urine is due primarily to the chloride content of the food ingested, it follows that a decrease in the amount of ingested chloride will likewise cause a decrease in the chloride content of the urine. In cases of actual fasting the chloride content of the urine may be decreased to *a slight trace* which is derived from the body fluids and tissues. Under these conditions, however, an examination of the blood of the fasting subject will show the percentage of chlorides in this fluid to be approximately normal. This forms a very striking example of the care nature takes to maintain the normal composition of the blood. There is a limit to the power of the body to maintain this equilibrium, however, and if the fasting organism be subjected to the influence of diuretics for a time, a point is reached where the composition of the blood can no longer be maintained and a gradual decrease in its chloride content occurs which finally results in death. Death is supposed to result not so much because of a lack of chlorine as from *a deficiency of sodium.* This is shown from the fact that potassium chloride, for instance, cannot replace the sodium chloride of the blood when the latter is decreased in the manner above stated. When this substitution is attempted the potassium salt is excreted at once in the urine, and death follows as above indicated.

Pathologically, the excretion of chlorides may be decreased in some fevers, chronic nephritis, croupous pneumonia, diarrhœa, certain stomach disorders, and in acute articular rheumatism.

Experiment.

Detection of Chlorides in Urine.—Place about 5 c.c. of urine in a test-tube, render it acid with nitric acid and add a few drops of a

solution of argentic nitrate. A white precipitate, due to the formation of argentic chloride, is produced. This precipitate is soluble in ammonium hydroxide.

Phosphates.

Phosphoric acid exists in the urine in two general forms: First, that in combination with the alkali metals, sodium and potassium, and the radical ammonium; second, that in combination with the alkaline earths, calcium and magnesium. Phosphates formed through a union of phosphoric acid with the alkali metals are termed *alkaline phosphates*, or phosphates of the alkali metals, whereas phosphates formed through a union of phosphoric acid with the alkaline earths are termed *earthy phosphates*, or phosphates of the alkaline earths.

Three series of salts are formed by phosphoric acid: *Normal*, M_3PO_4,[1] *mono-hydrogen*, M_2HPO_4, and *di-hydrogen*, MH_2PO_4. The di-hydrogen salts are acid in reaction, and it was generally believed that about 60 per cent of the total phosphate content of the urine was in the form of this type of salt, and that the acidity of the urine was due in great part to the presence of *sodium di-hydrogen phosphate*. Recently, however, it has been quite clearly shown that the normal acidity of the urine is not due to the presence of this salt, but is due, at least in part, to the presence of various acidic radicals. In this connection Folin believes that the phosphates in clear acid urine are *all* of the *mono-hydrogen* type, and that the acidity of the urines of this character is generally greater than the combined acidity of all the phosphates present; the excess in the acidity above that due to phosphates he believes to be due to *free organic acids*. The observation has recently been made that urine may be separated into two portions, one part consisting almost entirely of inorganic matter including practically *all of the phosphates* and having an *alkaline reaction*, the other containing practically all of the *organic substances* and no phosphates and having an *acid reaction*.

In bones the phosphates occur principally in the form of the normal salts of calcium and magnesium. The mono-hydrogen salts as a class are alkaline in reaction to litmus, and it is to the presence of di-sodium hydrogen phosphate, Na_2HPO_4, that the greater part of the alkalinity of the saliva is due.

The excretion of phosphoric acid is extremely variable, but on the average the total output for 24 hours is about 2.5 grams, expressed

[1] M may be occupied by any of the alkali metals or alkaline earths.

as P_2O_5. Ordinarily the total output is distributed between alkaline phosphates and earthy phosphates approximately in the ratio 2:1. The greater part of this phosphoric acid arises from the ingested food, either from the preformed phosphates or more especially from the phosphorus in organic combination such as we find it in *phosphoproteins*, *nucleoproteins* and *lecithins;* the phosphorus-containing tissues of the body also contribute to the total output of this element. Alkaline phosphates ingested with the food have a tendency to increase the phosphoric acid content of the urine to a greater extent than the earthy phosphates so ingested. This is due, in a measure, to the fact that a portion of the earthy phosphates, under certain conditions, may be precipitated in the intestine and excreted in the feces; this is especially to be noted in the case of herbivorous animals. Since the extent to which the phosphates are absorbed in the intestine depends upon the form in which they are present in the food, under ordinary conditions, there can be no absolute relationship between the urinary output of nitrogen and phosphorus. If the diet is constant, however, from day to day, thus allowing of the preparation of both a nitrogen and a phosphorus balance,[1] a definite ratio may be established. In experiments upon dogs, which were fed an exclusive meat diet, the ratio of nitrogen to phosphorus, in the urine and feces, was found to be 8.1 : 1.

Pathologically the excretion of phosphoric acid is increased in such diseases of the bones as diffuse periostosis, osteomalacia, and rickets; according to some investigators, in the early stages of pulmonary tuberculosis, in acute yellow atrophy of the liver, in diseases which are accompanied by an extensive decomposition of nervous tissue, and after sleep induced by potassium bromide or chloral hydrate (Mendel). It is also increased after copious water-drinking. A decrease in the excretion of phosphates is at times noted in febrile affections, such as the acute infectious diseases; in pregnancy, in the period during which the fœtal bones are forming, and in diseases of the kidneys, because of non-elimination.

Experiments.

1. **Formation of "Triple Phosphate."**—Place some urine in a beaker, render it alkaline with ammonium hydroxide, add a small amount of magnesium sulphate solution and allow the beaker to stand

[1] In metabolism experiments, a statement showing the relation existing between the nitrogen content of the food on the one hand and that of the urine and feces on the other, for a definite period, is termed a *nitrogen balance* or a "balance of the income and outgo of nitrogen."

in a cool place over night. Crystals of *ammonium magnesium phosphate*, "*triple phosphate*," form under these conditions. Examine the crystalline sediment under the microscope and compare the forms of the crystals with those shown in Fig. 96, below.

2. **"Triple Phosphate" Crystals in Ammoniacal Fermentation.**—Stand some urine aside in a beaker for several days. Ammoniacal fermentation will develop and "triple phosphate" crystals will form. Examine the sediment under the microscope and compare the crystals with those shown in Fig. 96, below.

FIG. 96.—"TRIPLE PHOSPHATE." (*Ogden.*)

3. **Detection of Earthy Phosphates.**—Place 10 c.c. of urine in a test-tube and render it alkaline with ammonium hydroxide. Warm the mixture and note the separation of a precipitate of *earthy phosphates*.

4. **Detection of Alkaline Phosphates.**—Filter off the earthy phosphates as formed in the last experiment, and add a small amount of magnesia mixture (see page 289) to the filtrate. Now warm the mixture and observe the formation of a white precipitate due to the presence of alkaline phosphates. Note the difference in the size of the precipitates of the two forms of phosphates from this same volume of urine. Which form of phosphates was present in the larger amount, *earthy* or *alkaline?*

5. **Influence upon Fehling's Solution.**—Place 2 c.c. of Fehling's solution in a test-tube, dilute it with 4 volumes of water and heat to boiling. Add a solution of sodium dihydrogen phosphate, NaH_2PO_4, a small amount at a time, and heat after each addition. What do you observe? What does this observation force you to conclude regarding the interference of phosphates in the testing of *diabetic* urine by means of Fehling's test?

Sodium and Potassium.

The elements sodium and potassium are always present in the urine. Usually they are combined with such acidic radicals as Cl, CO_3, SO_4 and PO_4. The amount of potassium, expressed as K_2O, excreted in 24 hours by an adult, subsisting upon a mixed diet, is on the average 2–3 grams, whereas the amount of sodium, expressed as Na_2O, under the same conditions, is ordinarily 4–6 grams. The ratio of K to Na is generally about 3 : 5. The absolute quantity of these elements excreted depends, of course, in large measure, upon the nature of the diet. Because of the non-ingestion of NaCl and the accompanying destruction of potassium-containing body tissues, the urine during fasting contains more potassium salts than sodium salts.

Pathologically the output of potassium, in its relation to sodium, may be increased during fever; following the crisis, however, the output of this element may be decreased. It may also be increased in conditions associated with *acid intoxication.*

Calcium and Magnesium.

The greater part of the calcium and magnesium excreted in the urine is in the form of phosphates. The daily output, which depends principally upon the nature of the diet, aggregates on the average about 1 gram and is made up of the phosphates of calcium and magnesium in the proportion of 1 : 2. The percentage of calcium salts present in the urine at any one time forms no dependable index as to the absorption of this class of salts, since they are again excreted into the intestine after absorption. It is therefore impossible to draw any satisfactory conclusions regarding the excretion of the alkaline earths unless we obtain accurate analytical data from both the feces and the urine.

Very little is known positively regarding the actual course of the excretion of the alkaline earths under pathological conditions except that an excess of calcium is found in *acid intoxication* and some diseases of the bones.

Carbonates.

Carbonates generally occur in small amount in the urine of man and carnivora under normal conditions, whereas much larger quantities are ordinarily present in the urine of herbivora. The alkaline reaction of the urine of herbivora is dependable in great measure upon the presence of carbonates. In general a urine containing carbonates

in appreciable amount is turbid when passed or becomes so shortly after. These bodies ordinarily occur as alkali or alkaline earth compounds and the turbid character of urine containing them is usually due principally to the latter class of substances. The carbonates of the alkaline earths are often found in amorphous urinary sediments.

Iron.

Iron is present in small amount in normal urine. It probably occurs partly in inorganic and partly in organic combination. The iron contained in urinary pigments or chromogens is in organic combination. According to different investigators the iron content of normal urine will probably not average more than 0.001 gram per day.

Experiment.

Detection of Iron in Urine.—Evaporate a convenient volume (10–15 c.c.) of urine to dryness. Incinerate and dissolve the residue in a few drops of iron-free hydrochloric acid and dilute the acid solution with 5 c.c. of water. Divide the acid solution into two parts and make the following tests: (*a*) To the first part add a solution of ammonium thiocyanate; a red color indicates the presence of iron. (*b*) To the second part of the solution add a little potassium ferrocyanide solution; a precipitate of Prussian blue forms upon standing.

Fluorides, Nitrates, Silicates and Hydrogen Peroxide.

These substances are all found in traces in human urine under normal conditions. Nitrates are undoubtedly introduced into the organism in the water and ingested food. The average excretion of nitrates is about 0.5 gram per day, the output being the largest upon a vegetable diet and smallest upon a meat diet. Nitrates are found only in urine which is undergoing decomposition and are formed from nitrates in the course of ammoniacal fermentation. Hydrogen peroxide has been detected in the urine, but its presence is believed to possess no pathological importance.

CHAPTER XIX.

URINE: PATHOLOGICAL CONSTITUENTS.[1]

- Dextrose.
- Proteins
  - Serum albumin.
  - Serum globulin.
  - Proteoses
    - Deutero-proteose.
    - Hetero-proteose.
    - "Bence-Jones' protein."
  - Peptone.
  - Nucleoprotein.
  - Fibrin.
  - Oxyhæmoglobin.
- Blood
  - Form elements.
  - Pigment.
- Bile
  - Pigments.
  - Acids.
- Acetone.
- Diacetic acid.
- β-Oxybutyric acid.
- Conjugate glycuronates.
- Pentoses.
- Fat.
- Hæmatoporphyrin.
- Lactose.
- Galactose.
- Lævulose.
- Inosite.
- Laiose.
- Melanin.
- Urorosein.
- Unknown substances.

DEXTROSE.

Traces of this sugar occur in normal urine, but the amount is not sufficient to be readily detected by the ordinary simple qualitative

[1] See note at the bottom of page 259.

tests. There are two distinct types of *pathological* glycosuria, *i. e.*, transitory glycosuria and persistent glycosuria. The transitory type may follow the ingestion of an excess of sugar, causing the *assimilation limit* to be exceeded, or it may accompany any one of several disorders which cause impairment of the power of assimilating sugar. In the persistent type large amounts of sugar are excreted daily in the urine for long periods of time. Under such circumstances a condition known as diabetes mellitus exists. Ordinarily, diabetic urine which contains a high percentage of sugar possesses a faint yellow color, a high specific gravity, and a volume which is above normal.

EXPERIMENTS.

1. **Phenylhydrazine Reaction.**—Test the urine according to one of the following methods: (*a*) To a small amount of phenylhydrazine mixture, furnished by the instructor,[1] add 5 c.c. of the urine, shake well, and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the tube to cool *slowly* and examine the crystals microscopically (Plate III, opposite page 23). If the solution has become too concentrated in the boiling process it will be light-red in color and no crystals will separate until it is diluted with water.

Yellow crystalline bodies called *osazones* are formed from certain sugars under these conditions, in general each individual sugar giving rise to an osazone of a definite crystalline form which is typical for that sugar. It is important to remember in this connection that, of the simple sugars of interest in physiological chemistry, dextrose and lævulose yield the same osazone, with phenylhydrazine. Each osazone has a definite melting-point, and as a further and more accurate means of identification it may be recrystallized and identified by the determination of its melting-point and nitrogen content. The reaction taking place in the formation of phenyldextrosazone is as follows:

$$\underset{\text{Dextrose.}}{C_6H_{12}O_6} + 2\underset{\text{Phenylhydrazine.}}{(H_2N.NH.C_6H_5)} = \underset{\text{Phenyldextrosazone.}}{C_6H_{10}O_4(N.NH.C_6H_5)_2} + 2H_2O + H_2.$$

(*b*) Place 5 c.c. of the urine in a test-tube, add 1 c.c. of phenylhydrazine-acetate solution furnished by the instructor,[2] and heat on a boiling water-bath for one-half to three-quarters of an hour. Allow the liquid to cool *slowly* and examine the crystals microscopically (Plate III, opposite p. 23).

[1] This mixture is prepared by combining one part of phenylhydrazine-hydrochloride and two parts of sodium acetate, by *weight*. These are thoroughly mixed in a mortar.

[2] This solution is prepared by mixing one part *by volume*, in each case, of glacial acetic acid, one part of water and two parts of phenylhydrazine (the base).

The phenylhydrazine test has been so modified by Cipollina as to be of use as a *rapid clinical test.* The directions for this test are given in the next experiment.

2. **Cipollina's Test.**—Thoroughly mix 4 c.c. of urine, 5 drops of phenylhydrazine (the base) and 1/2 c.c. of glacial acetic acid in a test-tube. Heat the mixture for about one minute over a low flame, shaking the tube continually to prevent loss of fluid by bumping. Add 4–5 drops of potassium hydroxide or sodium hydroxide (sp. gr. 1.16), being certain that the fluid in the test-tube remains acid; heat the mixture again for a moment and then cool the contents of the tube. Ordinarily the crystals form at once, especially if the urine possesses a low specific gravity. If they do not appear immediately allow the tube to stand at least 20 minutes before deciding upon the absence of sugar.

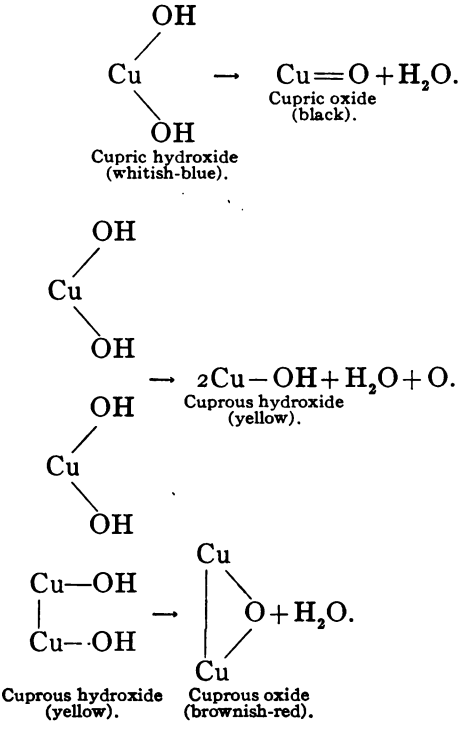
Examine the crystals under the microscope and compare them with those shown in Plate III, opposite page 23.

3. **Riegler's Reaction.**[1]—Introduce 0.1 gram of phenylhydrazine-hydrochloride and 0.25 gram of sodium acetate into a test-tube, add 20 drops of the urine under examination, and heat the mixture to boiling. Now introduce 10 c.c. of a 3 per cent solution of potassium hydroxide and gently shake the tube and contents. If the urine under examination contains dextrose the liquid in the tube will assume a red color. One per cent dextrose yields an immediate color whereas 0.05 per cent yields the color only after the lapse of a period of one-half hour from the time the alkali is added. If the color appears after the 30-minute interval the color change is without significance inasmuch as sugar-free urines will respond thus. The reaction is given by all aldehydes and therefore the test cannot be safely employed in testing urines preserved by formaldehyde. Albumin does not interfere with the test.

4. **Bottu's Test.**[2]—To 8 c.c. of Bottu's reagent[3] in a test-tube add 1 c.c. of the urine under examination and mix the liquids by gentle shaking. Now heat the upper portion of the mixture to boiling, add an additional 1 c.c. of urine and heat the mixture again immediately. The appearance of a blue color accompanied by the precipitation of small particles of indigo blue indicates the presence of dextrose in the urine under examination. The test will serve to detect the presence of 0.1 per cent of dextrose and is uninfluenced by creatinine or by ammonium salts.

[1] Riegler: *Compt. rend. soc.* biol., 66, p. 795.
[2] Bottu: *Compt. rend. soc.* biol., 66, p. 972.
[3] This reagent contains 3.5 grams of *o*-nitrophenylpropiolic acid and 5 c.c. of a freshly prepared 10 per cent. solution of sodium hydroxide per liter.

5. **Reduction Tests.**—To their aldehyde or ketone structure many sugars owe the property of readily reducing the alkaline solutions of the oxides of metals like copper, bismuth, and mercury; they also possess the property of reducing ammoniacal silver solutions with the separation of metallic silver. Upon this property of reduction the most widely used tests for sugars are based. When whitish-blue cupric hydroxide in suspension in an alkaline liquid is heated it is converted into insoluble black cupric oxide, but if a reducing agent like certain sugars be present the cupric hydroxide is reduced to insoluble yellow cuprous hydroxide, which in turn on further heating may be converted into brownish-red or red cuprous oxide. These changes are indicated as follows:



The chemical equations here discussed are exemplified in Trommer's and Fehling's tests.

(*a*) *Trommer's Test.*—To 5 c.c. of urine in a test-tube add one-half its volume of KOH or NaOH. Mix thoroughly and add, drop by drop, agitating after the addition of each drop, a *very dilute* solution of cupric sulphate. Continue the addition until there is a slight perma-

nent precipitate of cupric hydroxide and in consequence the solution is slightly turbid. Heat, and the cupric hydroxide is reduced to yellow cuprous hydroxide or to brownish-red cuprous oxide. If the solution of cupric sulphate used is too strong, a small brownish-red precipitate produced in the presence of a low percentage of dextrose may be entirely masked. On the other hand, if too little cupric sulphate is used a light-colored precipitate formed by uric acid and purine bases may obscure the brownish-red precipitate of cuprous oxide. The action of KOH or NaOH in the presence of an excess of sugar and insufficient copper will produce a brownish color. Phosphates of the alkaline earths may also be precipitated in the alkaline solution and be mistaken for cuprous hydroxide. Trommer's test is not very satisfactory.

(*b*) *Fehling's Test.*—To about 1 c.c. of Fehling's solution[1] in a test-tube add about 4 c.c. of water, and boil. This is done to determine whether the solution will of itself cause the formation of a precipitate of brownish-red cuprous oxide. If such a precipitate forms, the Fehling's solution must not be used. Add urine to the warm Fehling's solution, *a few drops* at a time, and heat the mixture after each addition. The production of yellow cuprous hydroxide or brownish-red cuprous oxide indicates that reduction has taken place. The yellow precipitate is more likely to occur if the urine is added rapidly and in large amount, whereas with a less rapid addition of smaller amounts of urine the brownish-red precipitate is generally formed.

This is a much more satisfactory test than Trommer's, but even this test is not entirely reliable when used to detect sugar in the urine. Such bodies as *conjugate glycuronates*, *uric acid*, *nucleoprotein*, and *homogentisic acid*, when present in sufficient amount, may produce a result similar to that produced by sugar. *Phosphates of the alkaline earths* may be precipitated by the alkali of the Fehling's solution and in appearance may be mistaken for the cuprous hydroxide. Cupric hydroxide may also be reduced to cuprous oxide and this in turn be dissolved by *creatinine*, a normal urinary constituent. This will give the urine under examination a greenish tinge and may obscure the sugar reaction even when a considerable amount of sugar is present.

(*c*) *Benedict's Modifications of Fehling's Test.—First Modification.*—

[1] Fehling's solution is composed of two definite solutions—a cupric sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

Cupric sulphate solution = 34.65 grams of cupric sulphate dissolved in water and made up to 500 c.c.

Alkaline tartrate solution = 125 grams of potassium hydroxide and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

To 2 c.c. of Benedict's solution[1] in a test-tube add 6 c.c. of distilled water and 7–9 drops (not more) of the urine under examination. Boil the mixture vigorously for about 15-30 seconds and permit it to cool to room temperature spontaneously. (If desired this process may be repeated, although it is ordinarily unnecessary.) If sugar is present in the solution a precipitate will form which is often *bluish-green* or *green* at first, especially if the percentage of sugar is low, and which usually becomes *yellowish* upon standing. If the sugar present exceeds 0.06 per cent this precipitate generally forms at or below the boiling-point, whereas if less than 0.06 per cent of sugar is present the precipitate forms more slowly and generally only after the solution has cooled. The greenish precipitate obtained with urines containing small amounts of sugar may be a compound of copper with the sugar or a compound of some constituent of the urine with reduced copper oxide instead of being a precipitate of cuprous hydroxide or oxide as is the case when the original Fehling solution is reduced.

Benedict claims that, whereas the original Fehling test will not serve to detect sugar when present in a concentration of less than 0.1 per cent, that the above modification will serve to detect sugar when present in as small quantity as 0.015–0.02 per cent. The modified solution used in the above test differs from the original in that 100 grams of sodium carbonate is substituted for the 125 grams of potassium hydroxide ordinarily used, thus forming a Fehling solution which is considerably *less alkaline* than the original. This alteration in the composition of the Fehling solution is of advantage in the detection of sugar in the urine inasmuch as the strong alkalinity of the ordinary Fehling solution has a tendency, when the reagent is boiled with a urine containing a small amount of dextrose, to decompose sufficient of the sugar to render the detection of the remaining portion exceedingly difficult by the usual technique. Benedict claims that for this reason the use of his modified solution permits the detection of smaller amounts of sugar than does the use of the ordinary Fehling solution. Benedict has further modified his solution for use in the quantitative determination of sugar (see page 363).

Second Modification.[2]—Very recently Benedict has further modi-

[1] Benedict's modified Fehling solution consists of two definite solutions—a cupric sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

Cupric sulphate solution = 34.65 grams of cupric sulphate dissolved in water and made up to 500 c.c.

Alkaline tartrate solution = 100 grams of anhydrous sodium carbonate and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

[2] Private communication from Dr. S. R. Benedict.

fied his solution and has succeeded in obtaining one which does not deteriorate upon long standing.[1] The following is the procedure for the detection of dextrose in the urine: To 5 c.c. of the reagent in a test-tube add eight (not more) drops of the urine to be examined. The fluid is then boiled vigorously for from one to two minutes and then allowed to cool *spontaneously*. In the presence of dextrose *the entire body of the solution will be filled* with a precipitate, which may be *red*, *yellow*, or *green* in color, depending upon the amount of sugar present. If no dextrose is present, the solution will either remain perfectly clear, or will show a very faint turbidity, due to precipitated urates. Even very small quantities of dextrose in urine (0.1 per cent) yield precipitates of surprising bulk with this reagent, and the positive reaction for dextrose is the filling of the entire body of the solution with a precipitate, so that the solution becomes opaque. Since *amount* rather than color of the precipitate is made the basis of this test, it may be applied, even for the detection of small quantities of dextrose, as readily in artificial light as in daylight.

(*d*) *Allen's Modification of Fehling's Test.*—The following procedure is recommended: "From 7 to 8 c.c. of the sample of urine to be tested is heated to boiling in a test-tube, and, without separating any precipitate of albumin which may be produced, 5 c.c. of the solution of cupric sulphate used for preparing Fehling's solution is added. This produces a precipitate containing uric acid, xanthine, hypoxanthine, phosphates, etc. To render the precipitation complete, however, it is desirable to add to the liquid, *when partially cooled*, from 1 to 2 c.c. of a saturated solution of sodium acetate having a feebly acid reaction to litmus.[2] The liquid is filtered and to the filtrate, which will have a bluish-green color, 5 c.c. of the alkaline tartrate mixture used for preparing Fehling's solution is added, and the liquid boiled for 15–20 seconds. In the presence of more than 0.25 per cent of sugar, separa-

[1] Benedict's new solution has the following composition:

Cupric sulphate	17.3 gm.
Sodium citrate	173.0 gm.
Sodium carbonate (anhydrous)	100.0 gm.
Distilled water to	1000.0 c.c.

With the aid of heat dissolve the sodium citrate and carbonate in about 600 c.c. of water. Pour (through a folded filter if necessary) into a glass graduate and make up to 850 c.c. Dissolve the cupric sulphate in about 100 c.c. of water and make up to 150 c.c. Pour the carbonate-citrate solution into a large beaker or casserole and add the cupric sulphate solution slowly, with constant stirring. The mixed solution is ready for use, and does not deteriorate upon long standing.

[2] Sufficient acetic acid should be added to the sodium acetate solution to render it feebly acid to litmus. A saturated solution of sodium acetate keeps well, but weaker solutions are apt to become mouldy, and then possess the power of reducing Fehling's solution. Hence it is essential in all cases of importance to make a blank test by mixing equal measures of cupric sulphate solution, alkaline tartrate solution and water, adding a little sodium acetate solution, and heating the mixture to boiling.

tion of cuprous oxide occurs before the boiling-point is reached; but with smaller quantities precipitation takes place during the cooling of the solution, which becomes greenish, opaque, and suddenly deposits cuprous oxide as a fine brownish-red precipitate."

(*e*) *Boettger's Test.*—To 5 c.c. of urine in a test-tube add 1 c.c. of KOH or NaOH and a very small amount of bismuth subnitrate, and boil. The solution will gradually darken and finally assume a black color due to reduced bismuth. If the test is made with urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced (bismuth sulphide).

(*f*) *Nylander's Test* (*Almén's Test*).—To 5 c.c. of urine in a test-tube add one-tenth its volume of Nylander's reagent[1] and heat for five minutes in a boiling water-bath.[2] The mixture will darken if reducing sugar is present and upon standing for a few moments a black color will appear. This color is due to the precipitation of bismuth. If the test is made on urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced. Dextrose when present to the extent of 0.08 per cent may be detected by this reaction. It is claimed by Bechold that Nylander's and Boettger's tests give a negative reaction with solutions containing sugar when mercuric chloride or chloroform is present. Other observers[3] have failed to verify the inhibitory action of the mercuric chloride and have shown that the inhibitory influence of chloroform may be overcome by raising the temperature of the urine to the boiling-point for a period of five minutes previous to making the test.

Urines rich in *indican*, *uroerythrin*, *urochrome* or *hæmatoporphyrin*, as well as urines excreted after the ingestion of large amounts of certain *medicinal substances*, may give a darkening of Nylander's reagent similar to that of a true sugar reaction. It is a disputed point whether the urine after the administration of urotropin will reduce Nylander's reagent.[4]

According to Rustin and Otto the addition of $PtCl_4$ increases the

[1] Nylander's reagent is prepared by digesting 2 grams of bismuth subnitrate and 4 grams of Rochelle salt in 100 c.c. of a 10 per cent potassium hydroxide solution. The reagent is then cooled and filtered.

[2] Hammarsten suggests that the solution be boiled for 2–5 minutes (according to the sugar content) over a free flame and the tube then permitted to stand five minutes before drawing conclusions.

[3] Rehfuss and Hawk: *Jour. Biol. Chem.*, VII, p. 267, 1910; also Zeidlitz: *Upsala Lakäreforen Forh.*, N. F., XI, 1906.

[4] Abt: *Archives of Pediatrics*, XXIV, p. 275, 1907; also Weitbrecht: *Schweiz. Woch.*, XLVII, p. 577, 1909.

delicacy of Nylander's reaction. They claim that this procedure causes the sugar to be converted *quantitatively*. No quantitative method has yet been devised, however, based upon this principle.

A positive Nylander or Boettger test is probably due to the following reactions:

$$(a) \qquad Bi(OH)_2(NO)_3 + KOH = Bi(OH)_3 + KNO_3.$$

$$(b) \qquad 2Bi(OH)_3 - 3O = Bi_2 + 3H_2O.$$

Bohmansson,[1] before testing the urine under examination treats it (10 c.c.) with 1/5 volume of 25 per cent hydrochloric acid and 1/2 volume of bone black. This mixture is shaken one minute, then filtered, and the neutralized filtrate tested by Nylander's reaction. Bahmansson claims that this procedure removes certain interfering substances, notably urochrome.

6. **Fermentation Test.**—Rub up in a mortar about 15 c.c. of the urine with a small piece of compressed yeast. Transfer the mixture to a saccharometer (Fig. 2, p. 31) and stand it aside in a warm place for about 12 hours. If dextrose is present, alcoholic fermentation will occur and carbon dioxide will collect as a gas in the upper portion of the tube. On the completion of fermentation introduce, by means of a bent pipette, a little KOH solution into the graduated portion, place the thumb tightly over the opening in the apparatus and invert the saccharometer. Explain the result.

7. **Barfoed's Test.**—Place about 5 c.c. of Barfoed's solution[2] in a test-tube and heat to boiling. Add the urine under examination slowly, a few drops at a time, heating after each addition. Reduction is indicated by the production of a red precipitate. If the precipitate does not form upon continued boiling allow the tube to stand a few minutes and examine. NaCl interferes with this test (Welker).

Barfoed's test is *not* a specific test for dextrose as is frequently stated, but simply serves to detect *monosaccharides*. Disaccharides will also respond to the test, according to Hinkel and Sherman, if the solution is boiled sufficiently long in contact with the reagent to *hydrolyze the disaccharide* through the action of the acetic acid present in the Barfoed's solution.

8. **Polariscopic Examination.**—For directions as to the use of the polariscope see page 31.

[1] Bohmansson: *Biochem. Zeit.*, 19, p. 281.

[2] Barfoed's solution is prepared as follows: Dissolve 4. 5 grams of neutral, crystallized cupric acetate in 100 c.c. of water and add 1.2 c.c. of 50 per cent acetic acid.

PROTEINS.

Normal urine contains a trace of protein material but the amount present is so slight as to escape detection by any of the simple tests in general use for the detection of protein urinary constituents. The following are the more important forms of protein material which have been detected in the urine under pathological conditions:

(1) Serum albumin.
(2) Serum globulin.
(3) Proteoses { Deutero-proteose. / Hetero-proteose. / "Bence-Jones' protein."
(4) Peptone.
(5) Nucleoprotein.
(6) Fibrin.
(7) Oxyhæmoglobin.

ALBUMIN.

Albuminuria is a condition in which serum albumin or serum globulin appears in the urine. There are two distinct forms of albuminuria, *i. e.*, *renal* albuminuria and *accidental* albuminuria. Sometimes the terms "true" albuminuria and "false" albuminuria are substituted for those just given. In the renal type the albumin is excreted by the kidneys. This is the more serious form of the malady and at the same time is more frequently encountered than the accidental type. Among the causes of renal albuminuria are altered blood pressure in the kidneys, altered kidney structure, or changes in the composition of the blood entering the kidneys, thus allowing the albumin to diffuse more readily. In the accidental form of albuminuria the albumin is *not* excreted by the kidneys as is the case in the renal form of the disorder, but arises from the blood, lymph, or some albumin-containing exudate coming into contact with the urine at some point below the kidneys.

EXPERIMENTS.

Heller's Ring Test.—Place 5 c.c. of concentrated HNO_3 in a test-tube, incline the tube, and, by means of a pipette allow the urine to flow slowly down the side. The liquids should stratify with the formation of a *white* zone of precipitated albumin at the point of juncture. If the albumin is present in very small amount the white

zone may not form until the tube has been allowed to stand for several minutes. If the urine is quite concentrated a white zone, due to uric acid or urates, will form upon treatment with nitric acid as indicated. This ring may be easily differentiated from the albumin ring by repeating the test after diluting the urine with 3 or 4 volumes of water, whereupon the ring, if due to uric acid or urates, will not appear. It is ordinarily possible to differentiate between the albumin ring and the uric acid ring without diluting the urine, since the ring, when due to uric acid, has ordinarily a less sharply defined upper border, is generally broader than the albumin ring and frequently is situated in the urine *above* the point of contact with the nitric acid. Concentrated urines also occasionally exhibit the formation, at the point of contact, of a *crystalline* ring with very sharply defined borders. This is urea nitrate and is easily distinguished from the "fluffy" ring of albumin. If there is any difficulty in differentiation a simple dilution of the urine with water, as above described, will remove the difficulty. Various colored zones, due either to the presence of indican, bile pigments, or to the oxidation of other organic urinary constituents, may form in this test under certain conditions. These colored rings should never be confounded with the *white* ring which alone denotes the presence of albumin.

After the administration of certain drugs a white precipitate of *resin acids* may form at the point of contact of the two fluids and may cause the observer to draw wrong conclusions. This ring, if composed of resin acids, will dissolve in alcohol, whereas the albumin ring will not dissolve.

Weinberger has recently shown that a ring closely resembling the albumin ring is often obtained in urines preserved by thymol when subjected to Heller's test. The ring is due to the formation of nitrosothymol and possibly nitrothymol. If the thymol is removed from the urine by extraction with petroleum ether[1] previous to adding nitric acid, the ring does not form.

An instrument called the *albumoscope* (*horismascope*) has been devised for use in this test and has met with considerable favor. The method of using the albumoscope is described below.

Use of the Albumoscope.—This instrument is intended to facilitate the making of "ring" tests such as Heller's and Roberts'. In making a test about 5 c.c. of the solution under examination is first introduced into the apparatus through the larger arm and the reagent used in the

[1] Accomplished readily by gently agitating equal volumes of petroleum ether and the urine under examination for *two minutes* in a test-tube before applying the test.

particular test is then introduced through the capillary arm and allowed to flow down underneath the solution under examination. If a reasonable amount of care is taken there is no possibility of mixing the two solutions and a definitely defined white "ring" is easily obtained at the zone of contact.

2. **Roberts' Ring Test.**—Place 5 c.c. of Roberts' reagent[1] in a test-tube, incline the tube, and, by means of a pipette, allow the urine to flow *slowly* down the side. The liquids should stratify with the formation of a *white* zone of precipitated albumin at the point of juncture. This test is a modification of Heller's ring test and is rather more satisfactory than that test, since the colored rings never form and the consequent confusion is avoided. The *albumoscope* (see above) may also be used in making this test.

3. **Spiegler's Ring Test.**—Place 5 c.c. of Spiegler's reagent[2] in a test-tube, incline the tube, and, by means of a pipette, allow 5 c.c. of urine, acidified with acetic acid, to flow *slowly* down the side. A white zone will form at the point of contact. This is an exceedingly delicate test, in fact too delicate for ordinary clinical purposes, since it serves to detect albumin when present in the merest trace (1 : 250,000) and hence most normal urines will give a positive reaction for albumin when this test is applied.

Some investigators claim that the delicacy of this test depends upon the presence of sodium chloride in the urine, the test losing accuracy if the sodium chloride content be low.

4. **Jolles' Reaction.**—Shake 5 c.c. of urine with 1 c.c. of 30 per cent acetic acid and 4 c.c. of Jolles' reagent[3] in a test-tube. A white precipitate indicates the presence of albumin.

Care should be taken to use the correct amount of acetic acid, since the use of too small an amount may result in the formation of mercury combinations which may cause confusion. In the presence of iodine, mercuric iodide will form but may readily be differentiated from albumin through the fact that it is *soluble* in alcohol.

5. **Coagulation or Boiling Test.**—(*a*) Heat 5 c.c. of urine to

[1] Roberts' reagent is composed of 1 volume of concentrated HNO_3 and 5 volumes of a saturated solution of $MgSO_4$.

[2] Spiegler's reagent has the following composition:

Tartaric acid	20 grams.
Mercuric chloride	40 grams.
Glycerol	100 grams.
Distilled water	1000 grams.

[3] Jolles' reagent has the following composition:

Succinic acid	40 grams.
Mercuric chloride	20 grams.
Sodium chloride	20 grams.
Distilled water	1000 grams.

boiling in a test-tube. A precipitate forming at this point is due either to albumin or to phosphates. Acidify the urine slightly by the addition of 3–5 drops of very dilute acetic acid, adding the acid drop by drop to the *hot* solution. If the precipitate is due to phosphates it will disappear under these conditions, whereas if it is due to albumin it will not only fail to disappear but will become more flocculent in character, since the reaction of a fluid must be acid to secure the complete precipitation of the albumin by this coagulation process. Too much acid should be avoided since it will cause the albumin to go into solution. Certain *resin acids* may be precipitated by the acid, but the precipitate due to this cause may be easily differentiated from the albumin precipitate by reason of its solubility in alcohol.

(*b*) A modification of this test in quite general use is as follows: Fill a test-tube two-thirds full of urine and gently heat the *upper half* of the fluid to boiling, being careful that this fluid does not mix with the lower half. A turbidity indicates albumin or phosphates. Acidify the urine slightly by the addition of 3–5 drops of dilute acetic acid, when the turbidity, if due to phosphates, will disappear.

Nitric acid is often used in place of acetic acid in these tests. In case nitric acid is used ordinarily 1–2 drops is sufficient.

6. **Acetic Acid and Potassium Ferrocyanide Test.**—To 5 c.c. of urine in a test-tube add 5–10 drops of acetic acid. Mix well and add potassium ferrocyanide *drop by drop*, until a precipitate forms. This is a very delicate test. Schmiedl claims that a precipitate of $Fe(Cn)_6K_2Zn$ or $Fe(Cn)_6Zn_2$ is formed when urines containing zinc are subjected to this test and that this precipitate resembles the precipitate secured with protein solutions. In the case of human urine a reaction was obtained when 0.000022 gram of zinc per cubic centimeter was present. Schmiedl further found that the urine collected from rabbits housed in zinc-lined cages possessed a zinc content which was sufficient to yield a ready response to the test. Zinc is the only interfering substance so far reported.

7. **Tanret's Test.**—To 5 c.c. of urine in a test-tube add Tanret's reagent[1] drop by drop until a turbidity or precipitate forms. This is an exceedingly delicate test. Sometimes the urine is stratified upon the reagent as in Heller's or Roberts' ring test. According to Repiton, urates interfere with the delicacy of this test. Tanret,

[1] Tanret's reagent is prepared as follows: Dissolve 1.35 gram of mercuric chloride in 25 c.c. of water, add to this solution 3.32 grams of potassium iodide dissolved in 25 c.c. of water, then make the total solution up to 60 c.c. with water and add 20 c.c. of glacial acetic acid to the mixture.

however, claims that urates do not interfere inasmuch as any precipitate due to urates may be brought into solution by heat whereas an albumin precipitate under the same conditions will persist. Tanret further states that *mucin* interferes with the delicacy of the test and that it should therefore be removed from the urine under examination by acidification with acetic acid and filtration before testing for albumin.

8. **Sodium Chloride and Acetic Acid Test.**—Mix two volumes of urine and one volume of a saturated solution of sodium chloride in a test-tube, acidify with acetic acid, and heat to boiling. The production of a cloudiness or the formation of a precipitate indicates the presence of albumin. The resin acids may interfere here as in the ordinary coagulation test (page 310), but they may be easily differentiated from albumin by means of their solubility in alcohol.

9. **Potassium Iodide Test.**[1]—Dilute 5 c.c. of the urine under examination with 10 c.c. of water and stratify this mixture upon a potassium iodide solution made slightly acid with acetic acid. In the presence of 0.01–0.02 per cent of albumin a white ring forms immediately. If the test be allowed to stand two minutes after the stratification it will serve to detect 0.005 per cent of albumin.

GLOBULIN.

Serum globulin is not a constituent of normal urine but frequently occurs in the urine under pathological conditions and is ordinarily associated with serum albumin. In albuminuria globulin in varying amounts often accompanies the albumin, and the clinical significance of the two is very similar. Under certain conditions globulin may occur in the urine unaccompanied by albumin.

EXPERIMENTS.

Globulin will respond to all the tests just outlined under Albumin. If it is desirable to differentiate between albumin and globulin in any urine the following processes may be employed:

1. **Saturation with Magnesium Sulphate.**—Place 25 c.c. of neutral urine in a small beaker and add pulverized magnesium sulphate *in substance* to the point of saturation. If the protein present is globulin it will precipitate at this point. If no precipitate is produced acidify the saturated solution with acetic acid and warm gently. Albumin will be precipitated if present.

[1] *Pharm. Ztg.*, 54, p. 612.

The above procedure may be used to separate globulin and albumin if present in the same urine. To do this filter off the globulin after it has been precipitated by the magnesium sulphate, then acidify the clear solution and warm gently as directed. Note the formation of the albumin precipitate.

2. **Half-saturation with Ammonium Sulphate.**—Place 25 c.c. of neutral urine in a small beaker and add an equal volume of a saturated solution of ammonium sulphate. Globulin, if present, will be precipitated. If no precipitate forms add ammonium sulphate *in substance* to the point of saturation. If albumin is present it will be precipitated upon saturation of the solution as just indicated. This method may also be used to separate globulin and albumin when they occur in the same urine.

Frequently in urine which contains a large amount of urates a precipitate of ammonium urate may occur when the ammonium sulphate solution is added to the urine. This urate precipitate should not be confounded with the precipitate due to globulin. The two precipitates may be differentiated by means of the fact that the urate precipitate ordinarily appears only after the lapse of several minutes whereas the globulin generally precipitates at once.

PROTEOSE AND PEPTONE.

Proteoses, particularly deutero-proteose and hetero-proteose, have frequently been found in the urine under various pathological conditions such as diphtheria, pneumonia, intestinal ulcer, carcinoma, dermatitis, osteomalacia, atrophy of the kidneys, and in sarcomata of the bones of the trunk. "Bence-Jones' protein," a proteose-like substance, is of interest in this connection and its appearance in the urine is believed to be of great diagnostic importance in cases of multiple myeloma or myelogenic osteosarcoma. By some investigators this protein is held to be a variety of hetero-proteose whereas others claim that it possesses albumin characteristics.

Peptone certainly occurs much less frequently as a constituent of the urine than does proteose, in fact most investigators seriously question its presence under any conditions. There are many instances of peptonuria cited in the early literature, but because of the uncertainty in the conception of what really constituted a peptone it is probable that in many cases of so-called peptonuria the protein present was really proteose.

EXPERIMENTS.

1. **Boiling Test.**—Make the ordinary coagulation test according to the directions given under Albumin, page 310. If no coagulable protein is found allow the boiled urine to stand and note the gradual appearance, in the cooled fluid, of a flaky precipitate of proteose. This is a crude test and should never be relied upon.

2. **Schulte's Method.**—Acidify 50 c.c. of urine with dilute acetic acid and filter off any precipitate of nucleoprotein which may form. Now test a few cubic centimeters of the urine for coagulable protein, by tests 2 and 5 under Albumin, p. 310. If coagulable protein is present remove it by coagulation and filtration before proceeding. Introduce 25 c.c. of the urine, freed from coagulable protein, into 150 c.c. of absolute alcohol and allow it to stand for 12–24 hours. Decant the supernatant fluid and dissolve the precipitate in a small amount of hot water. Now filter this solution, and after testing again for nucleoprotein with *very dilute* acetic acid, try the biuret test. If this test is positive the presence of proteose is indicated.[1]

Urobilin does not ordinarily interfere with this test since it is almost entirely dissolved by the absolute alcohol when the proteose is precipitated.

3. **v. Aldor's Method.**—Acidify 10 c.c. of urine with hydrochloric acid, add phosphotungstic acid until no more precipitate forms and centrifugate[2] the solution. Decant the supernatant fluid, add some absolute alcohol to the precipitate, and centrifugate again. This washing with alcohol is intended to remove the urobilin and hence should be continued so long as the alcohol exhibits any coloration whatever. Now suspend the precipitate in water and add potassium hydroxide to bring it into solution. At this point the solution may be blue in color, in which case decolorization may be secured by gently heating. Apply the biuret test to the *cool* solution. A positive biuret test indicates the presence of proteoses.

4. **Detection of "Bence-Jones' Protein."**—Heat the suspected urine very gently, carefully noting the temperature. At as low a temperature as 40° C. a turbidity may be observed, and as the temperature is raised to about 60° C. a flocculent precipitate forms and clings to the sides of the test-tube. If the urine is now acidified *very slightly* with acetic acid and the temperature further raised to 100° C.

[1] If it is considered desirable to test for peptone the proteose may be removed by saturation with $(NH_4)_2SO_4$ according to the directions given on page 112 and the filtrate tested for peptone by the biuret test.

[2] If not convenient to use a centrifuge the precipitate may be filtered off and washed on the filter paper with alcohol.

the precipitate at least partly disappears; it will return upon cooling the tube.

This property of precipitating at so low a temperature and of dissolving at a higher temperature is typical of "Bence-Jones' protein" and may be used to differentiate it from all other forms of protein material occurring in the urine.

NUCLEOPROTEIN.

There has been considerable controversy as to the proper classification for the protein body which forms the "nubecula" of normal urine. By different investigators it has been called *mucin*, *mucoid*, *phosphoprotein*, *nucleoalbumin*, and *nucleoprotein*. Of course, according to the modern acceptation of the meanings of these terms they cannot be synonymous. Mucin and mucoid are glycoproteins and hence contain no phosphorus (see p. 85), whereas phosphoproteins and nucleoproteins are phosphorized bodies. It may possibly be that both these forms of protein, *i. e.*, the glycoprotein and the phosphorized type, occur in the urine under certain conditions (see page 284). In this connection we will use the term *nucleoprotein*. The pathological conditions under which the content of nucleoprotein is increased includes all affections of the urinary passages and in particular pyelitis, nephritis, and inflammation of the bladder.

EXPERIMENTS.

1. **Detection of Nucleoprotein.**—Place 10 c.c. of urine in a small beaker, dilute it with three volumes of water to prevent precipitation of urates, and make the reaction *very strongly* acid with acetic acid. If the urine becomes turbid it is an indication that nucleoprotein is present.

If the urine under examination contains albumin the greater portion of this substance should be removed by boiling the urine before testing it for the presence of nucleoprotein.

2. **Ott's Precipitation Test.**—Mix 25 c.c. of the urine with an equal volume of a saturated solution of sodium chloride and slowly add Almén's reagent.[1] In the presence of nucleoprotein a voluminous precipitate forms.

BLOOD.

The pathological conditions in which blood occurs in the urine may be classified under the two divisions *hæmaturia* and *hæmoglobin-*

[1] Dissolve 5 grams of tannin in 240 c.c. of 50 per cent alcohol and add 10 c.c. of 25 per cent acetic acid.

uria. In hæmaturia we are able to detect not only the hæmoglobin but the unruptured corpuscles as well, whereas in hæmoglobinuria the pigment alone is present. Hæmaturia is brought about through blood passing into the urine because of some lesion of the kidney or of the urinary tract below the kidney. Hæmoglobinuria is brought about through hæmolysis, *i. e.*, the rupturing of the stroma of the erythrocyte and the liberation of the hæmoglobin. This may occur in scurvy, typhus, pyemia, purpura, and in other diseases. It may also occur as the result of a burn covering a considerable area of the body, or may be brought about through the action of certain poisons or by the injections of various substances having the power of dissolving the erythroctyes. Transfusion of blood may also cause hæmoglobinuria.

Experiments.

1. **Heller's Test.**—Render 10 c.c. of urine strongly alkaline with potassium hydroxide solution and heat to boiling. Upon allowing the heated urine to stand a precipitate of phosphates, colored red by the contained hæmatin, is formed. It is ordinarily well to make a "control" experiment using normal urine, before coming to a final decision.

Certain substances, such as cascara sagrada, rhubarb, santonin, and senna, cause the urine to give a similar reaction. Reactions due to such substances may be differentiated from the true blood reaction by the fact that both the precipitate and the pigment of the former reaction disappear when treated with acetic acid, whereas if the color is due to hæmatin the acid will only dissolve the precipitate of phosphates and leave the pigment undissolved.

2. **Teichmann's Hæmin Test.**—Place a small drop of the suspected urine or a small amount of the moist sediment on a microscopic slide, add a minute grain of sodium chloride and *carefully* evaporate to *dryness* over a *low* flame. Put a coverglass in place, run underneath it a drop of glacial acetic acid, and warm gently until the formation of gas bubbles is observed. Cool the preparation, examine under the microscope, and compare the form of the crystals with those reproduced in Figs. 58 and 59, page 194. (See Atkinson and Kendall's modification, p. 193.)

3. **Heller-Teichmann Reaction.**—Produce the pigmented precipitate according to directions given in Heller's test above. If there is a copious precipitate of phosphates and but little pigment the phosphates may be dissolved by treatment with acetic acid and

the residue used in the formation of the hæmin crystals according to directions in Experiment 2, p. 316.

4. **v. Zeynek and Nencki's Hæmin Test.**—To 10 c.c. of the urine under examination add acetone until no more precipitate forms. Filter off the precipitate and extract it with 10 c.c. of acetone rendered acid with 2–3 drops of hydrochloric acid. Place a drop of the resulting colored extract on a slide, immediately place a coverglass in position, and examine under the microscope. Compare the form of the crystals with those shown in Figs. 58 and 59, page 194. Hæmin crystals produced by this manipulation are sometimes very minute, thus rendering it difficult to determine the exact form of the crystal.

5. **Schalfijew's Hæmin Test.**—Place 20 c.c. of glacial acetic acid in a small beaker and heat to 80° C. Add 5 c.c. of the urine under examination, raise the temperature to 80 ° C., and stand the mixture aside to cool. Examine the crystals under the microscope and compare them with those shown in Figs. 58 and 59, page 194.

6. **Guaiac Test.**—Place 5 c.c. of urine in a test-tube and by means of a pipette introduce a freshly prepared alcoholic solution of guaiac (strength about 1:60) into the fluid until a turbidity results, then add old turpentine or hydrogen peroxide, drop by drop, until a blue color is obtained. This is a very delicate test when properly performed. Buckmaster has recently suggested the use of guaiaconic acid instead of the solution of guaiac. See discussion on page 188 and test on page 191.

7. **Schumm's Modification of the Guaiac Test.**—To about 5 c.c. of urine[1] in a test-tube add about 10 drops of a freshly prepared alcoholic solution of guaiac. Agitate the tube gently, add about 20 drops of old turpentine, subject the tube to a thorough shaking, and permit it to stand for about 2–3 minutes. A blue color indicates the presence of blood in the solution under examination. In case there is not sufficient blood to yield a blue color under these conditions, a few c.c. of alcohol should be added and the tube gently shaken, whereupon a blue coloration will appear in the upper alcohol-turpentine layer.

A control test should always be made using water in place of urine. In the detection of very minute traces of blood only 3–5 drops of the guaiac solution should be employed.

8. **Adler's Benzidine Reaction.**—This is one of the most delicate of the reactions for the detection of blood. Different benzidine preparations vary greatly in their sensitiveness, however. Inasmuch as

[1] Alkaline urine should be made slightly acid with acetic acid as the blue end-reaction is very sensitive to alkali.

benzidine solutions change readily upon contact with light, it is essential that they be kept in a dark place. The test is performed as follows: To a saturated solution of benzidine in alcohol or glacial acetic acid add an equal volume of 3 per cent hydrogen peroxide and 1 c.c. of the urine under examination. If the mixture is not already acid, render it so with acetic acid, and note the appearance of a green or blue color. A control test should be made substituting water for the urine.

Often when urines containing a small amount of blood are tested by this reaction, the mixture is rendered so turbid as to make it difficult to decide as to the presence of a faint green color. Such urines should be extracted with an ether-acetic acid solution and the resulting extract washed with water before the test is applied to it. The sensitiveness of the benzidine reaction is greater when applied to aqueous solutions than when applied to the urine.

9. **Spectroscopic Examination.**—Submit the urine to a spectroscopic examination according to the directions given on page 198, looking especially for the absorption-bands of oxyhæmoglobin and methæmoglobin (see Absorption Spectra, Plate I.).

BILE.

Both the pigments and the acids of the bile may be detected in the urine under certain pathological conditions. Of the pigments, bilirubin is the only one which has been positively identified in fresh urine; the other pigments, when present, are probably derived from the bilirubin. A urine containing bile may be yellowish-green to brown in color and when shaken foams readily. The staining of the various tissues of the body through the absorption of bile due to occlusion of the bile duct causes a condition known as icterus or jaundice. Bile is always present in the urine under such conditions unless the amount of bile reaching the tissues is extremely small.

EXPERIMENTS.

Tests for Bile Pigments.

1. **Gmelin's Test.**—To about 5 c.c. of *concentrated* nitric acid in a test-tube add an equal volume of urine *carefully* so that the two fluids do not mix. At the point of contact note the various colored rings, *green*, *blue*, *violet*, *red*, and *reddish-yellow*.

2. **Rosenbach's Modification of Gmelin's Test.**—Filter 5 c.c. of urine through a small filter paper. Introduce a drop of *concentrated*

nitric acid into the cone of the paper and observe the succession of colors as given in Gmelin's test.

3. **Nakayama's Reaction.**—To 5 c.c. of urine in a test-tube add an equal volume of a 10 per cent solution of barium chloride. Centrifugate the mixture, pour off the supernatant fluid, and heat the precipitate with 2 c.c. of Nakayama's reagent.[1] In the presence of bile pigments the solution assumes a blue or green color.

3. **Huppert's Reaction.**—Thoroughly shake equal volumes of urine and milk of lime in a test-tube. The pigments unite with the calcium and are precipitated. Filter off the precipitate, wash it with water, and transfer to a small beaker. Add alcohol acidified slightly with hydrochloric acid and warm upon a water-bath until the solution becomes colored an emerald green.

According to Steensma, this procedure may give negative results even in the presence of the pigments, owing to the fact that the acid-alcohol is not a sufficiently strong oxidizing agent. He therefore suggests the addition of a drop of a 0.5 per cent solution of sodium nitrite to the acid-alcohol mixture before warming on the water-bath. Try this modification also.

4. **Salkowski's Test.**—Render 5 c.c. of urine alkaline with a few drops of a 10 per cent sodium carbonate solution and add a 10 per cent solution of calcium chloride, drop by drop, until the supernatant fluid exhibits the normal urinary color when the contents of the test-tube are thoroughly mixed. Filter off the precipitate, and after washing it place it in a second tube with 95 per cent alcohol. Acidify the alcohol with hydrochloric acid and, if necessary, shake the tube to bring the precipitate into solution. Heat the solution to boiling and observe the appearance of a green color which changes through blue and violet to red; if no bile is present the solution does not undergo any color change. This test will frequently exhibit greater delicacy than Gmelin's test. Steensma's suggestions mentioned under Huppert's Reaction, above, apply in connection with this test also.

5. **Hammarsten's Reaction.**—To about 5 c.c. of Hammarsten's reagent[2] in a small evaporating dish add a few drops of urine. A green color is produced. If more of the reagent is now added the play of colors as noted in Gmelin's test may be obtained.

6. **Smith's Test.**—To 2–3 c.c. of urine in a test-tube *add carefully*

[1] Prepared by combining 99 c.c. of alcohol and 1 c.c. of fuming hydrochloric acid containing 4 grams of ferric chloride per liter.

[2] Hammarsten's reagent is made by mixing 1 volume of 25 per cent nitric acid and 19 volumes of 25 per cent. hydrochloric acid and then adding 1 volume of this acid mixture to 4 volumes of 95 per cent alcohol.

about 5 c.c. of dilute tincture of iodine (1:10) so that the fluids do not mix. A green ring is observed at the point of contact.

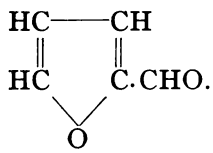
7. **Salkowski-Schippers Reaction.**—Neutralize the acidity of 10 c.c. of the urine under examination with a few drops of a dilute solution of sodium carbonate, and add 5 drops of a 20 per cent solution of sodium carbonate and 10 drops of a 20 per cent solution of calcium chloride. Filter off the resultant precipitate upon a hardened filter paper and wash it with water. Remove the precipitate to a small porcelain dish, add 3 c.c. of an acid-alcohol mixture[1] and a few drops of a dilute solution of sodium nitrite and heat. The production of a green color indicates the presence of bile pigments.

8. **Bonanno's Reaction.**[2]—Place 5–10 c.c. of the urine under examination in a small porcelain evaporating dish and add a few drops of Bonanno's reagent.[3] If bile is present an emerald-green color will develop. Bonanno says the reaction is not interfered with by any known normal or pathological urinary constituent.

Tests for Bile Acids.

1. **Pettenkofer's Test.**—To 5 c.c. of urine in a test-tube add 5 drops of a 5 per cent solution of sucrose. Now incline the tube, run about 2–3 c.c. of concentrated sulphuric acid *carefully* down the side and note the *red* ring at the point of contact. Upon slightly agitating the contents of the tube the whole solution gradually assumes a *reddish* color. As the tube becomes warm, it should be cooled in running water in order that the temperature may not rise above 70° C.

2. **Mylius's Modification of Pettenkofer's Test.**—To approximately 5 c.c. of urine in a test-tube add 3 drops of a very dilute (1:1000) aqueous solution of furfurol,



Now incline the tube, run about 2–3 c.c. of concentrated sulphuric acid carefully down the side and note the *red* ring as above. In this case also, upon shaking the tube, the whole solution is colored red. Keep the temperature below 70° C. as before.

[1] Made by adding 5 c.c. of concentrated hydrochloric acid to 95 c.c. of 96 per cent alcohol.

[2] Il Tommasi, 2, No. 21.

[3] This reagent may be prepared by dissolving 2 grams of sodium nitrite in 100 c.c. of concentrated hydrochloric acid.

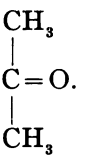
3. **Neukomm's Modification of Pettenkofer's Test.**—To a few drops of urine in an evaporating dish add a trace of a dilute sucrose solution and one or more drops of dilute sulphuric acid. Evaporate on a water-bath and observe the development of a *violet* color at the edge of the evaporating mixture. Discontinue the evaporation as soon as the color is observed.

4. **v. Udransky's Test.**—To 5 c.c. of urine in a test-tube add 3–4 drops of a very dilute (1 : 1000) aqueous solution of furfurol. Place the thumb over the top of the tube and shake until a thick foam is formed. By means of a small pipette add 2–3 drops of concentrated sulphuric acid to the foam and observe the *dark pink* coloration produced.

6. **Hay's Test.**—This test is based upon the principle that bile acids have the property of reducing the surface tension of fluids in which they are contained. The test is performed as follows: Cool about 10 c.c. of urine in a test-tube to 17° C. or lower, and sprinkle a little finely pulverized sulphur upon the surface of the fluid. The presence of bile acids is indicated if the sulphur sinks to the bottom of the liquid, the rapidity with which the sulphur sinks depending upon the amount of bile acids present in the urine. The test is said to react with bile acids when the latter are present in the proportion 1 : 120,000.

Some investigators claim that it is impossible to differentiate between bile acids and bile pigments by this test.

ACETONE,



It was formerly very generally believed that acetone appeared in the urine under pathological conditions because of increased protein decomposition. It is now generally thought that, in man, the output of acetone arises principally from the breaking down of fatty tissues or fatty foods within the organism. The quantity of acetone eliminated has been shown to increase when the subject is fed an abundance of fat-containing food as well as during fasting, whereas a replacement of the fat with carbohydrates is followed by a marked decrease in the acetone excretion. Conditions are different with certain of the lower animals. With the dog, for instance, the output of acetone is not diminished when the animal is fed upon a carbohydrate diet, is

decreased during fasting, and increased when the animal is fed upon a diet of meat.

Acetone and the closely related bodies, β-oxybutyric acid and diacetic acid, are generally classified as the *acetone bodies*. They are all associated with a deranged metabolic function and may appear in the urine together or separately, depending upon the conditions. Acetone and diacetic acid may occur alone in the urine but β-oxybutyric acid is never found except in conjunction with one or the other of these bodies. Acetone and diacetic acid arise chiefly from the oxidation of β-oxybutyric acid. The relation existing between these three bodies is shown in the following reactions:

(*a*) $\underset{\beta\text{-oxybutyric acid.}}{CH_3 \cdot CH(OH) \cdot CH_2 \cdot COOH} + O = \underset{\text{Diacetic acid.}}{CH_3CO \cdot CH_2 \cdot COOH} + H_2O.$

(*b*) $\underset{\text{Diacetic acid.}}{CH_3CO \cdot CH_2 \cdot COOH} = \underset{\text{Acetone.}}{(CH_3)_2CO} + CO_2.$

Acetone, chemically considered, is a ketone, *di-methyl ketone*. When pure it is a liquid which possesses a characteristic aromatic fruit-like odor, boils at 56–57° C. and is miscible with water, alcohol, or ether in all proportions. Acetone is a *physiological* as well as a pathological constituent of the urine and under normal conditions the daily output is about 0.01–0.03 gram.

Pathologically, the elimination of acetone is often greatly increased and at such times a condition of *acetonuria* is said to exist. This pathological acetonuria may accompany diabetes mellitus, scarlet fever, typhoid fever, pneumonia, nephritis, phosphorus poisoning, grave anæmias, fasting, and a deranged digestive function; it also frequently accompanies auto-intoxication and chloroform and ether anæsthesia. The types of acetonuria most frequentlv met with are those noted in febrile conditions and in advanced cases of diabetes mellitus.

Experiments.

1. **Isolation from the Urine.**—In order to facilitate the detection of acetone in the urine, the specimen under examination should be distilled and the tests as given below applied to the resulting distillate. If it is not convenient to distil the urine, the tests may be conducted upon the undistilled fluid. To obtain an acetone distillate proceed as follows: Place 100–250 c.c. of urine in a distillation flask or retort and render it acid with acetic acid. Collect about one-third of the orig-

inal volume of fluid as a distillate, add 5 drops of 10 per cent hydrochloric acid and redistil about one-half of this volume. With this final distillate conduct the tests as given below.

2. **Gunning's Iodoform Test.**—To about 5 c.c. of the urine or distillate in a test-tube add a few drops of Lugol's solution[1] or ordinary iodine solution (I in KI) and enough NH_4OH to form a black precipitate (nitrogen iodide). Allow the tube to stand (the length of time depending upon the content of acetone in the fluid under examination) and note the formation of a yellowish sediment consisting of iodoform. Examine the sediment under the microscope and compare the form of the crystals with those shown in Fig. 6, p. 42. If the crystals are not well formed recrystallize them from ether and examine again. The crystals of iodoform should not be confounded with those of stellar phosphate (Fig. 76, p. 220) which may be formed in this test, particularly if made upon the undistilled urine. This test is preferable to Lieben's test (4) since no substance other than acetone will produce iodoform when treated according to the directions for this test; both alcohol and aldehyde yield iodoform when tested by Lieben's test.

Gunning's test is rather the most satisfactory test yet suggested for the detection of acetone, and may be used with good results even upon the undistilled urine. In some instances where the amount of acetone present is very small it is necessary to allow the tube to stand 24 hours before making the examination for iodoform crystals. This test serves to detect acetone when present in the ratio 1 : 100,000.

3. **Legal's Test.**—Introduce about 5 c.c. of the urine or distillate into a test-tube, add a few drops of a freshly prepared aqueous solution of sodium nitroprusside and render the mixture alkaline with potassium hydroxide. A ruby red color, due to creatinine, a normal urinary constituent, is produced (see Weyl's test, p. 273). Add an excess of acetic acid and if acetone is present the red color will be intensified, whereas in the absence of acetone a yellow color will result. Make a control test upon normal urine to show that this is so. A similar red color may be produced by paracresol in urines containing no acetone.

4. **Lieben's Test.**—Introduce 5 c.c. of the urine or distillate into a test-tube, render it alkaline with potassium hydroxide and add 1–2 c.c. of iodine solution drop by drop. If acetone is present a yellowish precipitate of iodoform will be produced. Identify the iodoform by means of its characteristic odor and its typical crystalline form (see Fig. 6, p. 42). While fully as delicate as Gunning's test (2) this test

[1] Lugol's solution may be prepared by dissolving 4 grams of iodine and 6 grams of potassium iodide in 100 c.c. of distilled water.

is not as accurate since by means of the procedure involved, either alcohol or aldehyde will yield a precipitate of iodoform. This test is especially liable to lead to erroneous deductions when urines from the advanced stages of diabetes are under examination, because of the presence of alcohol formed from the sugar through fermentative processes.[1]

5. **Reynolds-Gunning Test.**—This test depends upon the solubility of mercuric oxide in acetone and is performed as follows: To 5 c.c. of the urine or distillate add a few drops of mercuric chloride, render the solution alkaline with potassium hydroxide and add an equal volume of 95 per cent alcohol. Shake thoroughly in order to bring the major portion of the mercuric oxide into solution and filter. Render the *clear* filtrate faintly acid with hydrochloric acid and stratify some ammonium sulphide, $(NH_4)_2S$, upon this acid solution. At the zone of contact a blackish-gray ring of precipitated mercuric sulphide, HgS, will form. Aldehyde also responds to this test. Aldehyde, however, has never been detected in the urine and could only be present in this instance if the acidified urine was distilled too far.

6. **Taylor's Test.**—To 10 c.c. of the urine or distillate in a test-tube add a few drops of a freshly prepared aqueous solution of sodium nitroprusside and stratify concentrated ammonium hydroxide upon the mixture. The production of a magenta color at the point of contact indicates the presence of acetone in the urine or distillate under examination. Normal urine yields an orange-red color when subjected to this technique.

DIACETIC ACID,

$$\begin{array}{l} CH_3 \\ | \\ C=O \\ | \\ CH_2 \cdot COOH. \end{array}$$

Diacetic or acetoacetic acid occurs in the urine only under pathological conditions and is rarely found except associated with acetone. It is formed from β-oxybutyric acid, another of the *acetone bodies*, and upon decomposition yields acetone and carbon dioxide. Diaceturia occurs ordinarily under the same conditions as the pathological acetonuria, *i. e.*, in fevers, diabetes, etc. (see p. 322). If very little diacetic acid is formed it may all be transformed into acetone, whereas if a

[1] Welker reports the production of a pink or red color during the application of this test to the distillates from pathological urines which had been preserved with powdered thymol. He found the color to be due to an iodothymol compound which had been previously prepared synthetically by Messinger and Vortmann.

larger quantity is produced both acetone and diacetic acid may be present in the urine. Diaceturia is most frequently observed in children, especially accompanying fevers and digestive disorders; it is perhaps less frequently observed in adults, but when present, particularly in fevers and diabetes, it is frequently followed by fatal coma.

Diacetic acid is a colorless liquid which is miscible with water, alcohol, and ether, in all proportions. It differs from acetone in giving a violet-red or Bordeaux-red color with a dilute solution of ferric chloride.

Experiments.

1. **Gerhardt's Test.**—To 5 c.c. of urine in a test-tube add ferric chloride solution, drop by drop, until no more precipitate forms. In the presence of diacetic acid a Bordeaux-red color is produced; this color may be somewhat masked by the precipitate of ferric phosphate, in which case the fluid should be filtered.

A positive result from the above manipulation simply indicates the *possible* presence of diacetic acid. Before making a final decision regarding the presence of this body make the two following control experiments:

(*a*) Place 5 c.c. of urine in a test-tube and boil it vigorously for 3–5 minutes. Cool the tube and, with the boiled urine, make the test as given above. As has been already stated, diacetic acid yields acetone upon decomposition and acetone does *not* give a Bordeaux-red color with ferric chloride. By boiling as indicated above, therefore, any diacetic acid present would be decomposed into acetone and carbon dioxide and the test upon the resulting fluid would be negative. If positive the color is due to the presence of bodies other than diacetic acid.

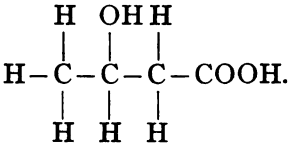
(*b*) Place 5 c.c. of urine in a test-tube, acidify with H_2SO_4, to free diacetic acid from its salts, and *carefully* extract the mixture with ether by shaking. If diacetic acid is present it will be extracted by the ether. Now remove the ethereal solution and add to it an equal volume of ferric chloride; diacetic acid is indicated by the production of the characteristic Bordeaux-red color. This color disappears spontaneously in 24–48 hours. Such substances as antipyrin, kairin, phenacetin, salicylic acid, salicylates, sodium acetate, thiocyanates, and thallin yield a similar red color under these conditions, but when due to the presence of any of these substances the color does not disappear spontaneously but may remain permanent for days. Many

of these disturbing substances are soluble in benzene or chloroform and may be removed from the urine by this means before extracting with ether as above. Diacetic acid is insoluble in benzene or chloroform.

2. **Arnold-Lipliawsky Reaction.**—This reaction is somewhat more delicate than Gerhardt's test (1) and serves to detect diacetic acid when present in the proportion of 1:25,000. It is also negative toward acetone, β-oxybutyric acid and the interfering drugs mentioned as causing erroneous deductions in the application of Gerhardt's test. If the urine under examination is highly pigmented it should be partly decolorized by means of animal charcoal before applying the test as indicated below.

Place 5 c.c. of the urine under examination and an equal volume of the Arnold-Lipliawsky reagent[1] in a test-tube, add a few drops of concentrated ammonia and shake the tube vigorously. Note the production of a brick-red color. Take 1–2 c.c. of this colored solution, add 10–20 c.c. of hydrochloric acid (sp. gr. 1.19), 3 c.c. of chloroform, and 2–4 drops of ferric chloride solution and carefully mix the fluids *without shaking*. Diacetic acid is indicated by the chloroform assuming a violet or blue color; if diacetic acid is absent the color may be yellow or light red.

β-OXYBUTYRIC ACID,



This acid does not occur as a normal constituent of urine but is found only under pathological conditions and then always in conjunction with either acetone or diacetic acid. Either of these bodies may be formed from β-oxybutyric acid under proper conditions. It is present in especially large amount in severe cases of diabetes and has also been detected in digestive disturbances, continued fevers, scurvy, measles, and in starvation. It is probable that, in man, β-oxybutyric acid, in common with acetone and diacetic acid, arises principally from the breaking down of fatty tissues within the organism.

[1] This reagent consists of two definite solutions which are ordinarily preserved separately and mixed just before using. The two solutions are prepared as follows:

(*a*) One per cent aqueous solution of potassium nitrite.

(*b*) One gram of *p*-amino-acetophenon dissolved in 100 c.c. of distilled water and enough hydrochloric acid (about 2 c.c.) added, drop by drop, to cause the solution, which is at first yellow, to become entirely colorless. An excess of acid must be avoided.

Before using, *a* and *b* are mixed in the ratio 1 : 2.

The condition in which large amounts of acetone and diacetic acid, and in severe cases β-oxybutyric acid also, are excreted in the urine is known as "acidosis." In diabetes the deranged metabolic conditions cause the production of great quantities of these substances which lead to an acid intoxication and ultimately to diabetic coma.

Ordinarily β-oxybutyric acid is an odorless, transparent syrup, which is lævorotatory and easily soluble in water, alcohol, and ether; it may be obtained in crystalline form.

Experiments.

1. **Black's Reaction.**—Inasmuch as the urinary pigments as well as any contained sugar or diacetic acid will interfere with the delicacy of this test when applied to the urine directly the following preliminary procedure is necessary: Concentrate 10 c.c. of the urine under examination to one-third or one-fourth of its original volume in an evaporating dish at a *gentle heat.* Acidify the residue with a few drops of concentrated hydrochloric acid, add sufficient plaster of Paris to make a thick paste and allow the mixture to stand until it begins to "set." It should now be stirred and broken up in the dish by means of a stirring rod with a blunt end. Extract the porous meal thus produced twice with ether by stirring and decantation. Any β-oxybutyric acid present will be extracted by the ether. Evaporate the ether extract spontaneously or on a water-bath; dissolve the residue in water, and neutralize it with barium carbonate. To 5 to 10 c.c. of this neutral fluid in a test-tube add two to three drops of ordinary commercial acid hydrogen peroxide. Mix by shaking and add a few drops of Black's reagent.[1] Permit the tube to stand and note the gradual development of a *rose color* which increases to its maximum intensity and then gradually fades.[2]

In carrying out the test care should be taken to see that the solution is *cold* and *approximately neutral* and that a *large excess* of hydrogen peroxide and Black's reagent are not added. In case but little β-oxybutyric acid is present the color will fail to appear or will be but transitory if the oxidizing agents are added in *too great excess.* It is preferable to add a few drops of the reagent and at intervals of a few minutes repeat the process until the color undergoes no further increase in intensity. One part of β-oxybutyric acid in 10,000 parts of the solution may be detected by this test.

[1] Made by dissolving 5 grams of ferric chloride and 0.4 gram of ferrous chloride in 100 c.c. of water.

[2] This disappearance of color is due to the further oxidation of the diacetic acid.

2. **Polariscopic Examination.**—Subject some of the urine (free from protein) to the ordinary fermentation test (see page 307). This will remove dextrose and lævulose, which would interfere with the polariscopic test. Now examine the fermented fluid in the polariscope and if it is lævorotatory the presence of β-oxybutyric acid is indicated. This test is not absolutely reliable, however, since conjugate glycuronates are also lævorotatory after fermentation.

3. **Kulz's Test.**—Evaporate the urine, after fermenting it as indicated in the last test, to a syrup, add an equal volume of concentrated sulphuric acid, and distil the mixture directly without cooling. Under these conditions α-crotonic acid is formed and is present in the distillate. Allow the distillate to cool slowly and note the formation of crystals of α-crotonic acid which are soluble in ether and melt at 72° C. In case very slight traces of β-oxybutyric acid be present in the urine under examination the amount of α-crotonic acid formed may be too small to yield a crystalline product. In this event the distillate should be extracted with ether, the ethereal extract evaporated, and the residue washed with water. Under these conditions the impurities will be removed and the α-crotonic acid will remain behind as a residue. The melting-point of this residue may then be determined.

CONJUGATE GLYCURONATES.

Glycuronic acid does not occur free in the urine, but is found, for the most part, in combination with phenol. Much smaller quantities are excreted in combination with indoxyl and skatoxyl. The total content of conjugate glycuronates seldom exceeds 0.004 per cent under normal conditions. The output may be very greatly increased as the result of the administration of antipyrin, borneol, camphor, chloral, menthol, morphine, naphthol, turpentine, etc. The glycuronates as a group are lævorotatory, whereas glycuronic acid is dextrorotatory. Most of the glycuronates reduce alkaline metallic oxides and so introduce an error in the examination of urine for sugar. Conjugate glycuronates often occur associated with dextrose in glycosuria, diabetes mellitus, and in some other disorders. As a class the glycuronates are non-fermentable.

Experiments.

1. **Fermentation-Reduction Test.**—Test the urine by Fehling's test. If there is reduction try Barfoed's test. If negative this indicates the absence of monosaccharides. A negative fermentation test would

now indicate the presence of conjugate glycuronates (or lactose in rare cases).[1]

If dextrose is present in the urine tested for glycuronates the urine must first be subjected to a polariscopic examination, then fermented and a second polariscopic examination made. The sugar being dextrorotatory and fermentable and the glycuronates being lævorotatory and non-fermentable the second polariscopic test will show a lævorotation indicative of conjugate glycuronates.

2. **Tollens' Reaction.**—Make this test according to directions given under Pentoses, below.

PENTOSES.

We have two distinct types of pentosuria, *i. e.*, *alimentary pentosuria*, resulting from the ingestion of large quantities of pentose-rich vegetables such as prunes, cherries, grapes, or plums, and fruit juices, in which condition the pentoses appear only *temporarily* in the urine; and the *chronic* form of pentosuria, in which the output of pentoses bears no relation whatever to the quantity and nature of the pentose content of the food eaten. In occurring in these two forms, pentosuria resembles glycosuria (see page 300), but it is definitely known that pentosuria bears no relation to diabetes mellitus and there is no generally accepted theory to account for the occurrence of the chronic form of pentosuria. The pentose detected most frequently in the urine is arabinose, the inactive form generally occurring in chronic pentosuria and the lævorotatory variety occurring in the alimentary type of the disorder.

Experiments.

1. **Tollens' Reaction.**—To equal volumes of urine and hydrochloric acid (sp. gr. 1.09) add a little phloroglucin and heat the mixture on a boiling water-bath. Pentose, galactose, or glycuronic acid will be indicated by the appearance of a red color. To differentiate between these bodies examine by the spectroscope and look for the absorption band between D and E given by pentoses and glycuronic acid, and then differentiate between the two latter bodies by the melting-points of their osazones.

2. **Orcin Test.**—Place equal volumes of urine and hydrochloric acid (sp. gr. 1.09) in a test-tube, add a small amount of orcin, and

[1] If necessary to differentiate between lactose and glycuronates apply the mucic acid test (see p. 40) or the phenylhydrazine reaction (see p. 23).

heat the mixture to boiling. Color changes from red through reddish-blue to green will be noted. When the solution becomes green it should be shaken in a separatory funnel with a little amyl alcohol, and the alcoholic extract examined spectroscopically. An absorption band between C and D will be observed.

FAT.

When fat finds its way into the urine through a lesion which brings some portion of the urinary passages into communication with the lymphatic system a condition known as *chyluria* is established. The turbid or milky appearance of such urine is due to its content of chyle. This disease is encountered most frequently in tropical countries, but is not entirely unknown in more temperate climates. Albumin is a constant constituent of the urine in chyluria. Upon shaking a chylous urine with ether the fat is dissolved by the ether and the urine becomes clearer or entirely clear.

HÆMATOPORPHYRIN.

Urine containing this body is occasionally met with in various diseases, but more frequently after the use of quinine, tetronal, trional, and especially sulphonal. Such urines ordinarily possess a reddish tint, the depth of color varying greatly under different conditions.

Experiments.

1. **Spectroscopic Examination.**—To 100 c.c. of urine add about 20 c.c. of a 10 per cent solution of potassium hydroxide or ammonium hydroxide. The precipitate which forms consists principally of earthy phosphates to which the hæmatoporphyrin adheres and is carried down. Filter off the precipitate, wash it and transfer to a flask and warm with alcohol acidified with hydrochloric acid. By this process the hæmatoporphyrin is dissolved and on filtering will be found in the filtrate and may be identified by means of the spectroscope (see page 202, and Absorption Spectra, Plate II).

2. **Acetic Acid Test.**—To 100 c.c. of urine add 5 c.c. of glacial acetic acid and allow the mixture to stand 48 hours. Hæmatoporphyrin deposits in the form of a precipitate.

LACTOSE.

Lactose is rarely found in the urine except as it is excreted by women during pregnancy, during the nursing period, or soon after weaning.

It is rather difficult to show the presence of lactose in the urine in a satisfactory manner, since the formation of the characteristic lactosazone is not attended with any great measure of success under these conditions. It is, however, comparatively easy to show that it is not dextrose, for, while it responds to reduction tests, it does not ferment with *pure* yeast and does not give a dextrosazone. An absolutely conclusive test, of course, is the isolation of the lactose in crystalline form (Fig. 75, p. 215) from the urine.

On oxidation with nitric acid lactose and galactose yield *mucic acid.* This test is frequently used in urine examination to differentiate lactose and galactose from other reducing sugars.

Experiments.

1. **Mucic Acid Test.**—Treat 100 c.c. of the urine under examination with 20 c.c.[1] of concentrated nitric acid and evaporate the mixture in a broad, shallow glass vessel, upon a boiling water-bath until the volume of the solution is only about 20 c.c. At this point the fluid should be clear and a fine white precipitate of *mucic acid* should separate. If the percentage of lactose in the urine is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will form. It is impossible to differentiate between galactose and lactose by means of this test, but the reaction does serve to differentiate these two sugars from all other reducing sugars. A satisfactory differentiation between lactose and galactose may be made by means of Barfoed's test, p. 30.

2. **Rubner's Test.**—To 10 c.c. of urine in a small beaker add some plumbic acetate, in substance, heat to boiling, and add NH_4OH until no more precipitate is dissolved. In the presence of lactose a brick-red or rose-red color develops, whereas dextrose gives a coffee-brown color, maltose a light yellow color, and lævulose no color at all under the same conditions.

3. **Compound Test.**—Try the phenylhydrazine test, the fermentation test, and Barfoed's test according to directions given under Dextrose, pages 23, and 30. If these are negative, try Nylander's test, page 29. If this last test is positive, the presence of lactose is indicated.

[1] If the specific gravity of the urine is 1020 or over it is necessary to use 25–35 c.c. of nitric acid. Under these conditions the mixture should be evaporated until the remaining volume is approximately equivalent to that of the nitric acid added.

GALACTOSE.

Galactose has occasionally been detected in the urine, and in particular in that of nursing infants afflicted with a deranged digestive function. Lactose and galactose may be differentiated from other reducing sugars which may be present in the urine by means of the mucic acid test. This test simply consists in the production of mucic acid through oxidation of the sugar with nitric acid.

EXPERIMENTS.

1. **Mucic Acid Test.**—Treat 100 c.c. of the urine under examination with 20 c.c.[1] of concentrated nitric acid and evaporate the mixture in a broad, shallow glass vessel, upon a boiling water-bath, until the volume of the solution is only about 20 c.c. At this point the fluid should be clear and a fine, white precipitate of *mucic acid* should separate. If the percentage of galactose present in the urine is low it may be necessary to cool the solution and permit it to stand for some time before the precipitate will form. It is impossible to differentiate between galactose and lactose by means of this test, but the reaction does serve to differentiate these two sugars from all other reducing sugars. A satisfactory differentiation between galactose and lactose may be made by Barfoed's test, p. 30.

2. **Tollens' Reaction.**—To equal volumes of the urine and hydrochloric acid (sp. gr. 1.09) add a little phloroglucin and heat the mixture on a boiling water-bath. Galactose, pentose, and glycuronic acid will be indicated by the appearance of a red color. Galactose may be differentiated from the two latter substances in that its solutions exhibit no absorption bands upon spectroscopical examination.

LÆVULOSE.

Diabetic urine frequently possesses the power of rotating the plane of polarized light to the left, thus indicating the presence of a lævorotatory substance. This lævorotation is sometimes due to the presence of lævulose, although not necessarily confined to this carbohydrate, since conjugate glycuronates and β-oxybutyric acid, two other lævorotatory bodies, are frequently found in the urine of diabetics. Lævulose is invariably accompanied by dextrose in diabetic urine,

[1] If the specific gravity of the urine is 1020 or over it is necessary to use 25–35 c.c. of nitric acid. Under these conditions the mixture should be evaporated until the remaining volume is approximately equivalent to that of the nitric acid added.

but *lævulosuria* has been observed as a separate anomaly. The presence of lævulose may be inferred when the percentage of sugar, as determined by the titration method, is greater than the percentage indicated by the polariscopic examination.

Experiments.

1. **Borchardt's Reaction.**—To about 5 c.c. of urine in a test-tube add an equal volume of 25 per cent hydrochloric acid and a few crystals of resorcin. Heat to boiling and after the production of a red color, cool the tube under running water and transfer to an evaporating dish or beaker. Make the mixture slightly alkaline with *solid* potassium hydroxide, return it to a test-tube, add 2–3 c.c. of acetic ether, and shake the tube vigorously. In the presence of lævulose the acetic ether is colored *yellow*.

The only urinary constituents which interfere with the test are *nitrites* and *indican* and these interfere only when they are *simultaneously present*. Under these conditions, the urine should be acidified with acetic acid and heated to boiling for one minute to remove the nitrites. In case the indican content is *very large*, it will impart a *blue* color to the acetic ether, thus masking the yellow color due to lævulose. When such urines are to be examined, the indican should first be removed by Obermayer's test (see p. 275). The chloroform should then be discarded, the acid-urine mixture diluted with one-third its volume of water, and the test applied as described above. The urine of patients who have ingested *santonin* or *rhubarb* respond to the test. The test will serve to detect lævulose when present in a dilution of 1 : 2000, *i. e.*, 0.05 per cent.

2. **Seliwanoff's Reaction.**—To 5 c.c. of Seliwanoff's reagent[1] in a test-tube add a few drops of the urine under examination and heat the mixture to boiling. The presence of lævulose is indicated by the production of a red color and the separation of a red precipitate. The latter may be dissolved in alcohol to which it will impart a striking red color.

If the boiling be *prolonged* a similar reaction may be obtained with urines containing dextrose.

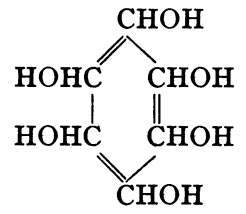
3. **Phenylhydrazine Test.**—Make the test according to directions under Dextrose, 3, page 23.

4. **Polariscopic Examination.**—A simple polariscopic examination, when taken in connection with other ordinary tests, will fur-

[1] Seliwanoff's reagent may be prepared by dissolving 0.05 gram of resorcin in 100 c.c. of dilute (1 : 2) hydrochloric acid.

nish the requisite data regarding the presence of lævulose, provided lævulose is not accompanied by other lævorotatory substances, such as conjugate glycuronates and β-oxybutyric acid.

INOSITE,



Inosite occasionally occurs in the urine in albuminuria, diabetes mellitus, and diabetes insipidus. It is claimed also that copious water-drinking causes this substance to appear in the urine. Inosite was at one time considered to be a sugar but is now known to be hexahydroxybenzene, as the above formula indicates. It is an example of a non-carbohydrate in whose molecule the H and O are present in the proportion to form water. In other words it has the formula of the hexoses, *i. e.*, $C_6H_{12}O_6$. Inosite occurs widely distributed in the vegetable kingdom, and because of this fact the theory has been voiced that it represents one of the first stages in the conversion of a carbohydrate into the benzene ring. It is found in the liver, spleen, lungs, brain, kidneys, suprarenal capsules, muscles, leucocytes, testes, and urine under normal conditions.

EXPERIMENT.

1. **Detection of Inosite.**—Acidify the urine with concentrated nitric acid and evaporate nearly to dryness. Add a few drops of ammonium hydroxide and a little calcium chloride solution to the moist residue and evaporate the mixture to dryness. In the presence of inosite (0.001 gram) a bright red color is obtained.

LAIOSE.

This substance is *occasionally* found in the urine in severe cases of diabetes mellitus. By some investigators laiose is classed with the sugars. It resembles lævulose in that it has the property of reducing certain metallic oxides and is lævorotatory, but differs from lævulose in being amorphous, non-fermentable, and in not possessing a sweet taste.

MELANINS.

These pigments never occur normally in the urine, but are present under certain pathological conditions, their presence being especially associated with melanotic tumors. Ordinarily the freshly passed urine is clear, but upon exposure to the air the color deepens and may at last be very dark brown or black in color. The pigment is probably present in the form of a chromogen or melanogen and upon coming in contact with the air oxidation occurs, causing the transformation of the melanogen into melanin and consequently the darkening of the urine.

It is claimed that melanuria is proof of the formation of a visceral melanotic growth. In many instances, without doubt, urines rich in indican have been wrongly taken as diagnostic proof of melanuria. The pigment melanin is sometimes mistaken for indigo and melanogen for indican. It is comparatively easy to differentiate between indigo and melanin through the solubility of the former in chloroform.

In rare cases melanin is found in urinary sediment in the form of fine amorphous granules.

Experiments.

1. **Zeller's Test.**—To 50 c.c. of urine in a small beaker add an equal volume of bromine water. In the presence of melanin a yellow precipitate will form and will gradually darken in color, ultimately becoming black.

2. **von Jaksch-Pollak Reaction.**—Add a few drops of ferric chloride solution to 10 c.c. of urine in a test-tube and note the formation of a gray color. Upon the further addition of the chloride a dark precipitate forms, consisting of phosphates and adhering melanin. An excess of ferric chloride causes the precipitate to dissolve.

This is the most satisfactory test for the identification of melanin in the urine.

UROROSEIN.

This is a pigment which is not present in normal urine but may be detected in the urine of various diseases, such as pulmonary tuberculosis, typhoid fever, nephritis, and stomach disorders. Urorosein, in common with various other pigments, does not occur preformed in the urine, but is present in the form of a chromogen, which is transformed into the pigment upon treatment with a mineral acid.

EXPERIMENTS.

1. **Robin's Reaction.**—Acidify 10 c.c. of urine with about 15 drops of concentrated hydrochloric acid. Upon allowing the acidified urine to stand, a rose-red color will appear if urorosein is present.

2. **Nencki and Sieber's Reaction.**—To 100 c.c. of urine in a beaker add 10 c.c. of 25 per cent sulphuric acid. Allow the acidified urine to stand and note the appearance of a rose-red color. The pigment may be separated by extraction with amyl alcohol.

UNKNOWN SUBSTANCES.

Ehrlich's Diazo Reaction.—Place equal volumes of urine and Ehrlich's diazobenzenesulphonic acid reagent[1] in a test-tube, mix thoroughly by shaking, and quickly add ammonium hydroxide in excess. The test is positive if both the fluid and the foam assume a red color. If the tube is allowed to stand a precipitate forms, the upper portion of which exhibits a blue, green, greenish-black, or violet color. Normal urine gives a brownish-yellow reaction with the above manipulation.

The exact nature of the substance or substances upon whose presence in the urine this reaction depends is not well understood. Some investigators claim that a positive reaction indicates an abnormal decomposition of protein material, whereas others assume it to be due to an increased excretion of alloxyproteic acid, oxyproteic acid, or uroferric acid.

The reaction may be taken as a metabolic symptom of certain disorders, which is of value diagnostically *only* when taken in connection with the other symptoms. The reaction appears principally in the urine in febrile disorders and in particular in the urine in typhoid fever, tuberculosis, and measles. The reaction has also been obtained in the urine in various other disorders such as carcinoma, chronic rheumatism, diphtheria, erysipelas, pleurisy, pneumonia, scarlet fever, syphilis, typhus, etc. The administration of alcohol, chrysarobin, creosote, cresol, dionin, guaiacol, heroin, morphine, naphthalene,

[1] Two separate solutions should be prepared and mixed in definite proportions when needed for use.

(*a*) Five grams of sodium nitrite dissolved in 1 liter of distilled water.

(*b*) Five grams of sulphanilic acid and 50 c.c. of hydrochloric acid in 1 liter of distilled water.

Solutions *a* and *b* should be preserved in well stoppered vessels and mixed in the proportion 1 : 50 when required. Green asserts that greater delicacy is secured by mixing the solutions in the proportion 1 : 100. The sodium nitrite deteriorates upon standing and becomes unfit for use in the course of a few weeks.

opium, phenol, tannic acid, etc., will also cause the urine to give a positive reaction.

The following chemical reactions take place in this test:

(*a*) $NaNO_2 + HCl = HNO_2 + NaCl.$

(*b*) $C_6H_4(NH_2)(HSO_3) + HNO_2 = C_6H_4(N{=}N)(SO_3) + 2H_2O.$

Sulphanilic acid. Diazo-benzenesulphonic acid.

CHAPTER XX.

URINE: ORGANIZED AND UNORGANIZED SEDIMENTS.

THE data obtained from carefully conducted microscopical examinations of the sediment of certain pathological urines are of very great importance, diagnostically. Too little emphasis is sometimes placed upon the value of such findings.

FIG. 97.—THE PURDY ELECTRIC CENTRIFUGE.

FIG. 98.—SEDIMENT TUBE FOR THE PURDY ELECTRIC CENTRIFUGE.

The sedimentary constituents may be divided into two classes, *i. e., organized* and *unorganized.* The sediment is ordinarily collected for examination by means of the centrifuge (Fig. 97, above). An older method, and one still in vogue in some quarters, is the so-called *gravity* method. This simply consists in placing the urine in a conical glass and allowing the sediment to settle. The collection of the sediment by means of the centrifuge, however, is much preferable, since

the process of sedimentation may be accomplished by the use of this instrument in a few minutes, and far more perfectly, whereas when the other method is used it is frequently necessary to allow the urine to remain in the conical glass 12–24 hours before sufficient sediment can be secured for the microscopical examination.

(a) Unorganized Sediments.

Ammonium magnesium phosphate ("Triple phosphate").
Calcium oxalate.
Calcium carbonate.
Calcium phosphate.
Calcium sulphate.
Uric acid.
Urates.
Cystine.
Cholesterol.
Hippuric acid.
Leucine and tyrosine.
Hæmatoidin and bilirubin.
Magnesium phosphate.
Indigo.
Xanthine.
Melanin.

Ammonium Magnesium Phosphate ("Triple Phosphate").—Crystals of "triple phosphate" are a characteristic constituent of the sediment when alkaline fermentation of the urine has taken place either *before* or after being voided. They may even be detected in amphoteric or *slightly* acid urine provided the ammonium salts are present in large enough quantity. This substance may occur in the sediment in two forms, *i. e.*, prisms and the feathery type. The prismatic form of crystals (Fig. 96, p. 296) is the one most commonly observed in the sediment; the feathery form (Fig. 96, p. 296) predominates when the urine is made ammoniacal with ammonia.

The sediment of the urine in such disorders as are accompanied by a retention of urine in the lower urinary tract contains "triple phosphate" crystals as a characteristic constituent. The crystals are frequently abundant in the sediment during paraplegia, chronic cystitis, enlarged prostate, and chronic pyelitis.

Calcium Oxalate.—Calcium oxalate is found in the urine in the form of at least two distinct types of crystals, *i. e.*, the *dumb-bell* type

and the *octahedral type* (Fig. 99, below). Either form may occur in the sediment of neutral, alkaline, or acid urine, but both forms are found most frequently in urine having an acid reaction. Occasionally, in alkaline urine, the octahedral form is confounded with "triple phosphate" crystals. They may be differentiated from the phosphate crystals by the fact that they are insoluble in acetic acid.

The presence of calcium oxalate in the urine is not of itself a sign of any abnormality, since it is a constituent of normal urine. It is

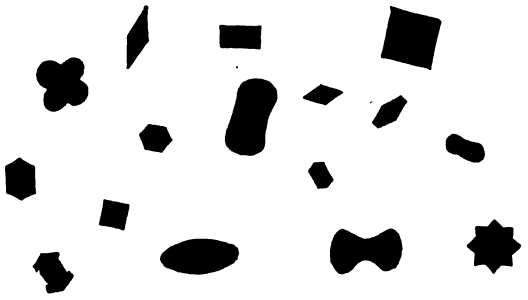


FIG. 99.—CALCIUM OXALATE. (*Ogden.*)

increased above the normal, however, in such pathological conditions as diabetes mellitus, in organic diseases of the liver, and in various other conditions which are accompanied by a derangement of digestion or of the oxidation mechanism, such as occurs in certain diseases of the heart and lungs.

Calcium Carbonate.—Calcium carbonate crystals form a typical constituent of the urine of herbivorous animals. They occur less frequently in human urine. The reaction of urine containing these crystals is nearly always alkaline, although they may occur in amphoteric or in *slightly* acid urine. It generally crystallizes in the form of granules, spherules, or dumb-bells (Fig. 100, p. 341). The crystals of calcium carbonate may be differentiated from calcium oxalate by the fact that they dissolve in acetic acid with the evolution of carbon dioxide gas.

Calcium Phosphate (Stellar Phosphate).—Calcium phosphate may occur in the urine in three forms, *i. e.*, amorphous, granular, or crystalline. The crystals of calcium phosphate are ordinarily pointed, wedge-shaped formations which may occur as individual crystals, or grouped together in more or less regularly formed rosettes (Fig. 76, p. 220). Acid sodium urate crystals (Fig. 102, p. 343) are o ten mistaken for crystals of calcium phosphate. We may differentiate between these two crystalline forms by the fact that acetic acid will readily dis-

solve the phosphate, whereas the urate is much less soluble and when finally brought into solution and recrystallized one is frequently enabled to identify uric acid crystals which have been formed from the acid urate solution. The clinical significance of the occurrence of calcium phosphate crystals in the urinary sediment is similar to that of "triple phosphate" (see page 296).

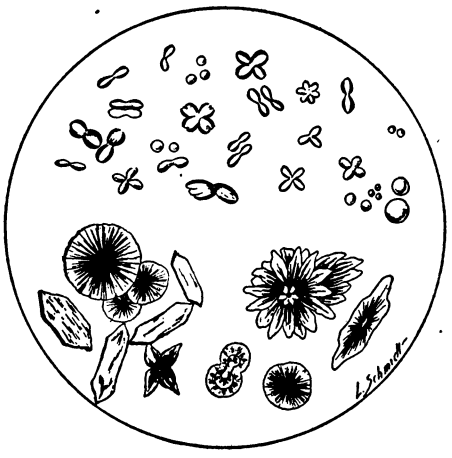


FIG. 100.—CALCIUM CARBONATE.

Calcium Sulphate.—Crystals of calcium sulphate are of quite rare occurrence in the sediment of urine. Their presence seems to be limited in general to urines which are of a decided acid reaction. Ordinarily it crystallizes in the form of long, thin, colorless needles or prisms (Fig. 95, page 292) which may be mistaken for calcium phosphate crystals. There need be no confusion in this respect, however, since the sulphate crystals are insoluble in acetic acid, which reagent readily dissolves the phosphate. As far as is known their occurrence as a constituent of urinary sediment is of very little clinical significance.

Uric Acid.—Uric acid forms a very common constituent of the sediment of urines which are acid in reaction. It occurs in more varied forms than any of the other crystalline sediments (Plate V, opposite page 267, and Fig. 101, page 342), some of the more common varieties of crystals being rhombic prisms, wedges, dumb-bells, whetstones, prismatic rosettes, irregular or hexagonal plates, etc. Crystals of pure uric acid are always colorless (Fig. 89, page 269), but the form occurring in urinary sediments is impure and under the microscope appears pigmented, the depth of color varying from light yellow to a dark reddish-brown according to the size and form of the crystal.

The presence of a considerable uric acid sediment does not, of necessity, indicate a pathological condition or a urine of increased uric acid content, since this substance very often occurs as a sediment in urines whose uric acid content is diminished from the normal merely as a result of changes in reaction, etc. Pathologically, uric acid sediments occur in gout, acute febrile conditions, chronic interstitial nephritis, etc. If the microscopical examination is not conclusive, uric acid may be differentiated from other crystalline urinary sediments from

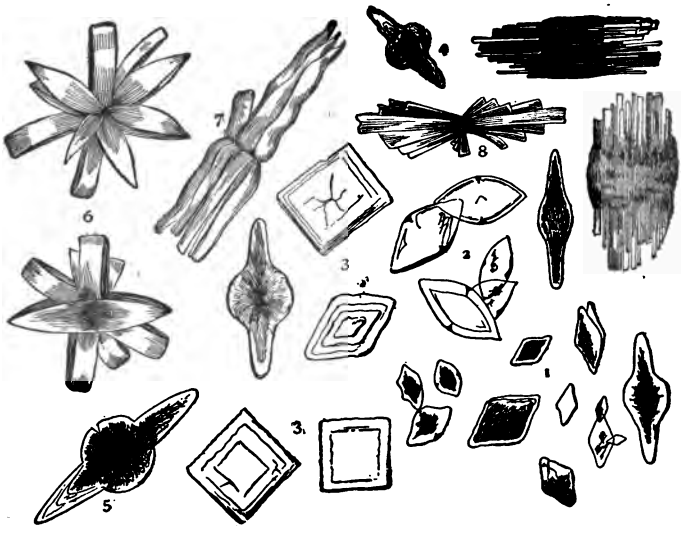


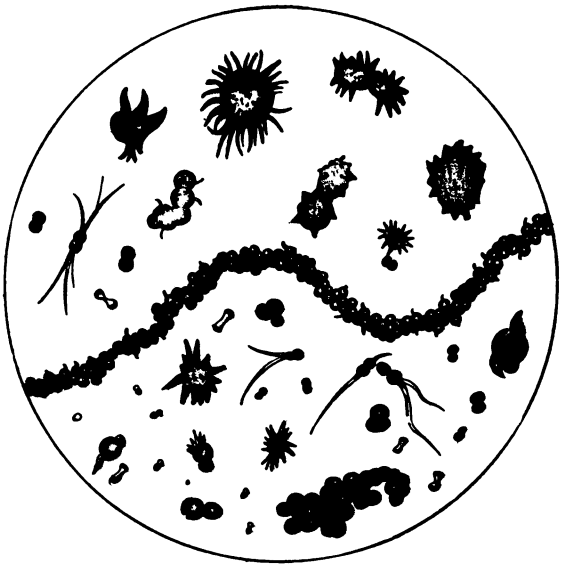
FIG. 101.—VARIOUS FORMS OF URIC ACID.

1, Rhombic plates; 2, whetstone forms; 3, 3, quadrate forms; 4, 5, prolonged into points; 6, 8, rosettes; 7, pointed bundles; 9, barrel forms precipitated by adding hydrochloric acid to urine.

the fact that it is soluble in alkalis, alkali carbonates, boiling glycerol, concentrated sulphuric acid, and in certain organic bases such as ethylamine and piperidin. It also responds to the murexide test (see page 269), Schiff's reaction (see page 269) and to Moreigne's reaction (see p. 269).

Urates.—The urate sediment may consist of a mixture of the urates of ammonium, calcium, magnesium, potassium, and sodium. The ammonium urate may occur in neutral, alkaline, or acid urine, whereas the other forms of urates are confined to the sediments of acid urines. Sodium urate occurs in sediments more abundantly than the other urates. The urates of calcium, magnesium, and potassium are amorphous in character, whereas the urate of ammonium is crystalline. Sodium urate may be either amorphous or crystalline. When crystal-

PLATE VI.



AMMONIUM URATE, SHOWING SPHERULES AND THORN-APPLE-SHAPED CRYSTALS.
(From *Odgen*, after *Peyer*.)

line it forms groups of fan-shaped clusters or colorless prismatic needles (Fig. 102, below). Ammonium urate is ordinarily present in the sediment in the burr-like form of the "thorn-apple" crystal, *i. e.*, yellow or reddish-brown spheres, covered with sharp spicules or prisms (Plate VI, opposite). The urates are all soluble in hydro-

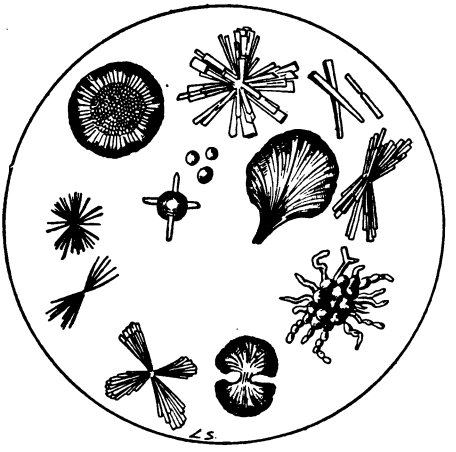


FIG. 102.—ACID SODIUM URATE.

chloric acid or acetic acid and their acid solutions yield crystals of uric acid upon standing. They also respond to the murexide test. The clinical significance of urate sediments is very similar to that of uric acid. A considerable sediment of amorphous urates does not necessarily indicate a high uric acid content, but ordinarily signifies a concentrated urine having a very strong acidity.

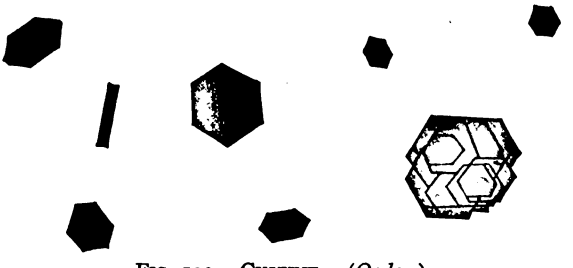


FIG. 103.—CYSTINE. (*Ogden.*)

Cystine.—Cystine is one of the rarer of the crystalline urinary sediments. It has been claimed that it occurs more often in the urine of men than of women. Cystine crystallizes in the form of thin, colorless, hexagonal plates (Fig. 24, p. 71, and Fig. 103, above) which are insoluble in water, alcohol, and acetic acid, and soluble in minerals acids,

alkalis, and especially in ammonia. Cystine may be identified by burning it upon platinum foil, under which condition it does not melt but yields a bluish-green flame.

Cholesterol.—Cholesterol crystals have been but rarely detected in urinary sediments. When present they probably arise from a pathological condition of some portion of the urinary tract. Crystals of cholesterol have been found in the sediment in cystitis, pyelitis, chyluria, and nephritis. Ordinarily it crystallizes in large regular and irregular colorless, transparent plates, some of which possess notched corners (Fig. 42, page 155). Frequently, instead of occurring in the sediment, it is found in the form of a film on the surface of the urine.

Hippuric Acid.—This is one of the rarer sediments of human urine. It deposits under conditions similar to those which govern the formation of uric acid sediments. The crystals, which are colorless needles or prisms (Fig. 92, page 276) when pure, are invariably pigmented in a manner similar to the uric acid crystals when observed in urinary sediment and because of this fact are frequently confounded with the rarer forms of uric acid. Hippuric acid may be differentiated from uric acid from the fact that it does not respond to the murexide test and is much more soluble in water and in ether. The detection of crystals of hippuric acid in the urine has very little clinical significance, since its presence in the sediment depends in most instances very greatly upon the nature of the diet. It is particularly prone to occur in the sediment after the ingestion of certain fruits as well as after the ingestion of benzoic acid (see page 276).

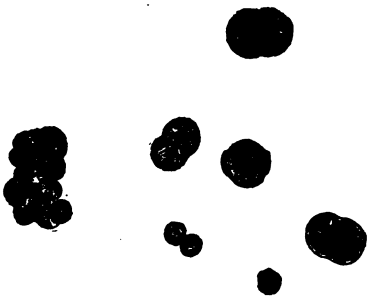


FIG. 104.—CRYSTALS OF IMPURE LEUCINE. (*Ogden.*)

Leucine and Tyrosine.—Leucine and tyrosine have frequently been detected in the urine, either in solution or as a sediment. Neither of them occurs in the urine ordinarily except in association with the other, *i. e.*, whenever leucine is detected it is more than probable that tyrosine accompanies it. They have been found pathologically in the urine in acute yellow atrophy of the liver, in acute phosphorus poisoning, in cirrhosis of the liver, in severe cases of typhoid fever and smallpox, and in leukæmia. In urinary sediments leucine ordinarily crystallizes in characteristic spherical masses which show both radial and concentric striations and are highly refractive (Fig. 104, above). Some investigators claim that these crystals which are ordinarily called leucine

are, in reality, generally urates. For the crystalline form of pure leucine obtained as a decomposition product of protein see Fig. 26, p. 75. Tyrosine crystallizes in urinary sediments in the well-known sheaf or tuft formation (Fig. 23, p. 71). For other tests on leucine and tyrosine see pages 81 and 82.

Hæmatoidin and Bilirubin.—There are divergent opinions regarding the occurrence of these bodies in urinary sediment. Each of them crystallizes in the form of tufts of small needles or in the form of small plates which are ordinarily yellowish-red in color (Fig. 41, p. 150). Because of the fact that the crystalline form of the two substances is identical many investigators claim them to be one and the same body. Other investigators claim, that while the crystalline form is the same in each case, there are certain chemical differences which may be brought out very strikingly by properly testing. For instance, it has been claimed that hæmatoidin may be differentiated from bilirubin through the fact that it gives a momentary color reaction (blue) when nitric acid is brought in contact with it, and, further, that it is not dissolved on treatment with ether or potassium hydroxide. Pathologically, typical crystals of hæmatoidin or bilirubin have been found in the urinary sediment in jaundice, acute yellow atrophy of the liver, carcinoma of the liver, cirrhosis of the liver, and in phosphorus poisoning, typhoid fever, and scarlatina.

Magnesium Phosphate.—Magnesium phosphate crystals occur rather infrequently in the sediment of urine which is neutral, alkaline, or *feebly* acid in reaction. It ordinarily crystallizes in elongated, highly refractive, rhombic plates which are soluble in acetic acid.

Indigo.—Indigo crystals are frequently found in urine which has undergone alkaline fermentation. They result from the breaking down of indoxyl-sulphates or indoxyl-glycuronates. Ordinarily indigo deposits as dark blue stellate needles or occurs as amorphous particles or broken fragments. These crystalline or amorphous forms may occur in the sediment or may form a blue film on the surface of the urine. Indigo crystals generally occur in urine which is alkaline in reaction, but they have been detected in acid urine.

Xanthine.—Xanthine is a constituent of normal urine but is found in the sediment in crystalline form very infrequently, and then only in pathological urine. When present in the sediment xanthine generally occurs in the form of whetstone-shaped crystals somewhat similar in form to the whetstone variety of uric acid crystal. They may be differentiated from uric acid by the great ease with which they may be brought into solution in dilute ammonia and on applying heat. Xanthine

may also form urinary calculi. The clinical significance of xanthine in urinary sediment is not well understood.

Melanin.—Melanin is an extremely rare constituent of urinary sediments. Ordinarily in melanuria the melanin remains in solution; if it separates it is generally held in suspension as fine amorphous granules.

(b) Organized Sediments.

Epithelial cells.
Pus cells.
Casts. { Hyaline. Granular. Epithelial. Blood. Fatty. Waxy. Pus.
Cylindroids.
Erythrocytes.
Spermatozoa.
Urethral filaments.
Tissue débris.
Animal parasites.
Micro-organisms.
Fibrin.
Foreign substances due to contamination.

Epithelial Cells.—The detection of a certain number of these cells in urinary sediment is not, of itself, a pathological sign, since they occur in normal urine. However, in certain pathological conditions they are greatly *increased* in number, and since different areas of the urinary tract are lined with different forms of epithelial cells, it becomes necessary, when examining urinary sediments, to note not only the relative number of such cells, but at the same time to carefully observe the shape of the various individuals in order to determine, as far as possible, from what portion of the tract they have been derived. Since the different layers of the epithelial lining are composed of cells different in form from those of the associated layers, it is evident that a careful microscopical examination of these cells may tell us the particular layer which is being desquamated. It is frequently a most difficult undertaking, however, to make a clear differentiation between the various

forms of epithelial cells present in the sediment. If skilfully done, such a microscopical differentiation may prove to be of very great diagnostic aid.

The principal forms of epithelial cells met with in urinary sediments are shown in Fig. 105, below.

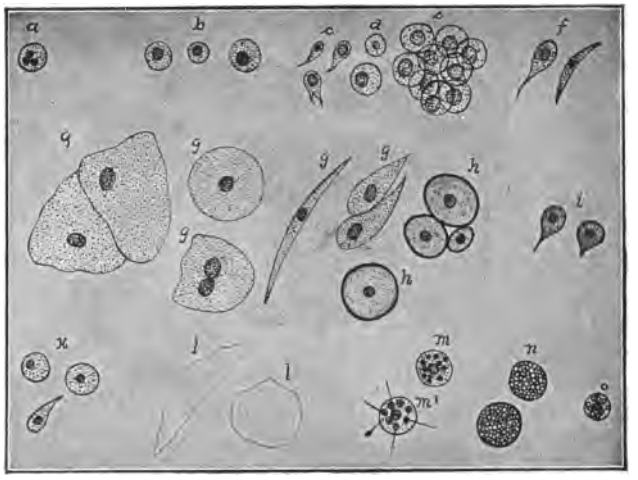


FIG. 105.—EPITHELIUM FROM DIFFERENT AREAS OF THE URINARY TRACT.

a, Leucocyte (for comparison); *b*, renal cells; *c*, superficial pelvic cells; *d*, deep pelvic cells; *e*, cells from calices; *f*, cells from ureter; *g*, *g*, *g*, *g*, *g*, squamous epithelium from the bladder; *h*, *h*, neck-of-bladder cells; *i*, epithelium from prostatic urethra; *k*, urethral cells; *l*, *l*, scaly epithelium; *m*, *m'*, cells from seminal passages; *n*, compound granule cells; *o*, fatty renal cell. (*Ogden.*)

Pus Cells.—Pus corpuscles or leucocytes are present in extremely small numbers in normal urine. Any considerable increase in the number, however, ordinarily denotes a pathological condition, generally an acute or chronic inflammatory condition of some portion of the urinary tract. The sudden appearance of a large amount of pus in a sediment denotes the opening of an abscess into the urinary tract. Other form elements, such as epithelial cells, casts, etc., ordinarily accompany pus corpuscles in urinary sediment and a careful examination of these associated elements is necessary in order to form a correct diagnosis as to the origin of the pus. Protein is always present in urine which contains pus.

The appearance which pus corpuscles exhibit under the microscope depends greatly upon the reaction of the urine containing them. In acid urine they generally present the appearance of round, colorless cells composed of refractive, granular protoplasm, and may frequently exhibit amœboid movements, especially if the slide containing them

be warmed slightly. They are nucleated (one or more nuclei), the nuclei being clearly visible only upon treating the cells with water, acetic acid, or some other suitable reagent. In urine which has a decided alkaline reaction, on the other hand, the pus corpuscles are often greatly degenerated. They may be seen as swollen, transparent cells, which exhibit no granular structure and as the process of degeneration continues the cell outline ceases to be visible, the nuclei fade, and finally only a mass of débris containing isolated nuclei and an occasional cell remains.

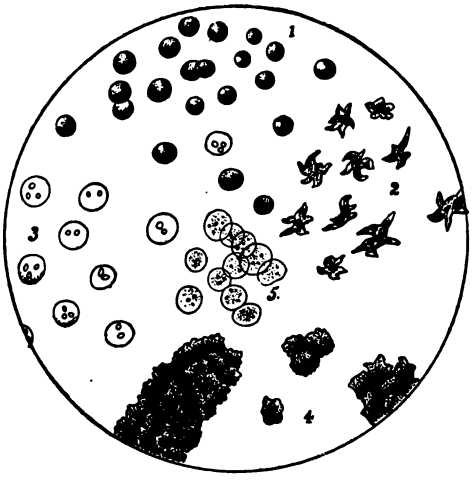


FIG. 106.—PUS CORPUSCLES. (After *Ultzmann.*)
1, Normal; 2, showing amœboid movements; 3, nuclei rendered distinct by acetic acid; 4, as observed in chronic pyelitis; 5, swollen by ammonium carbonate.

It is frequently rather difficult to make a differentiation between pus corpuscles and certain types of epithelial cells which are similar in form. Such confusion may be avoided by the addition of iodine solution (I in KI), a reagent which stains the pus corpuscles a deep mahogany-brown and transmits to the epithelial cells a light yellow tint. The test proposed by Vitali often gives very satisfactory results. This simply consists in acidifying the urine (if alkaline) with acetic acid, then filtering, and treating the sediment on the filter paper with freshly prepared tincture of guaiac. The presence of pus in the sediment is indicated if a *blue* color is observed. Large numbers of pus corpuscles are present in the urinary sediment in gonorrhœa, leucorrhœa, chronic pyelitis, and in abscess of the kidney.

Casts.—These are cylindrical formations, which originate in the uriniferous tubules and are forced out by the pressure of the urine.

They vary greatly in size, but in nearly every instance they possess parallel sides and rounded ends. The finding of casts in the urine is very important because of the fact that they *generally indicate* some kidney disorder; if albumin accompanies the casts the indication is much accentuated. Casts have been classified according to their microscopical characteristics as follows: (*a*) Hyaline, (*b*) granular, (*c*) epithelial, (*d*) blood, (*e*) fatty, (*f*) waxy, (*g*) pus.

(*a*) *Hyaline Casts.*—These are composed of a basic material which is transparent, homogeneous, and very light in color (Fig. 107, below).

FIG. 107.—HYALINE CASTS.
One cast is impregnated with four renal cells.

In fact, chiefly because of these physical properties, they are the most difficult form of renal casts to detect under the microscope. Frequently such casts are impregnated with deposits of various forms, such as erythrocytes, epithelial cells, fat globules, etc., thus rendering the form of the cast more plainly visible. Staining is often resorted to in order to render the shape and character of the cast more easily determined. Ordinary iodine solution (I in KI) may be used in this connection; many of the aniline dyes are also in common use for this purpose, *e. g.*, gentian-violet, Bismarck-brown, methylene-blue, fuchsin, and eosin. Generally, but not always, albumin is present in urine containing hyaline casts. Hyaline casts are common to all kidney disorders, but

occur particularly in the earliest and recovering stages of parenchymatous nephritis and interstitial nephritis.

(*b*) *Granular Casts.*—The common hyaline material is ordinarily the basic substance of this form of cast. The granular material generally consists of albumin, epithelial cells, fat, or disintegrated erythrocytes or leucocytes, the character of the cast varying according to the nature and size of the granules (Fig. 108,below, and Fig. 109, page 351). Thus we have casts of this general type classified as *finely granular* and *coarsely granular* casts. Granular casts, and in particular

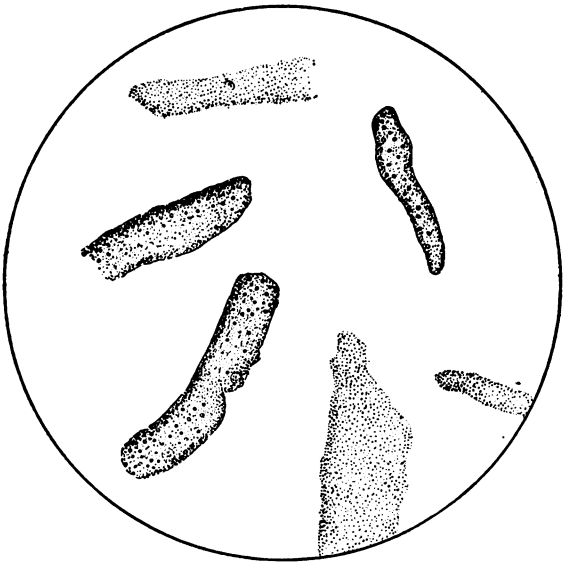


FIG. 108.—GRANULAR CASTS. (After *Peyer.*)

the finely granular types, occur in the sediment in practically every kidney disorder but are probably especially characteristic of the sediment in inflammatory disorders.

(*c*) *Epithelial Casts.*—These are casts bearing upon their surface epithelial cells from the lining of the uriniferous tubules (Fig. 110, p. 351). The basic material of this form of cast may be hyaline or granular in nature. Epithelial casts are particularly abundant in the urinary sediment in *acute* nephritis.

(*d*) *Blood Casts.*—Casts of this type may consist of erythrocytes borne upon a hyaline or a fibrinous basis (Fig. 111, p. 351). The occurrence of such casts in the urinary sediment denotes renal hemorrhage and they are considered to be especially characteristic of acute diffuse nephritis and acute congestion of the kidney.

(*e*) *Fatty Casts.*—Fatty casts may be formed by the deposition of fat globules or crystals of fatty acid upon the surface of a hyaline or granular cast (Fig. 112, p. 352). In order to constitute a true fatty

FIG. 109.—GRANULAR CASTS.
a, Finely granular; *b*, coarsely granular.

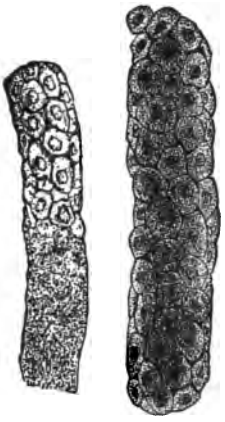


FIG. 110.—EPITHELIAL CASTS.

cast the deposited material must cover the greater part of the surface area of the cast. The presence of fatty casts in urinary sediment indicates fatty degeneration of the kidney; such casts are particularly characteristic of subacute and chronic inflammations of the kidney.

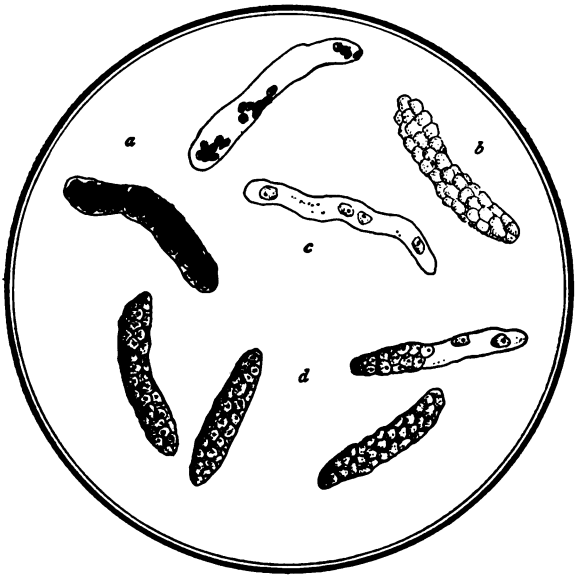


FIG. 111.—BLOOD, PUS, HYALINE AND EPITHELIAL CASTS.
a, Blood casts; *b*, pus cast; *c*, hyaline cast impregnated with renal cells; *d*, epithelial casts.

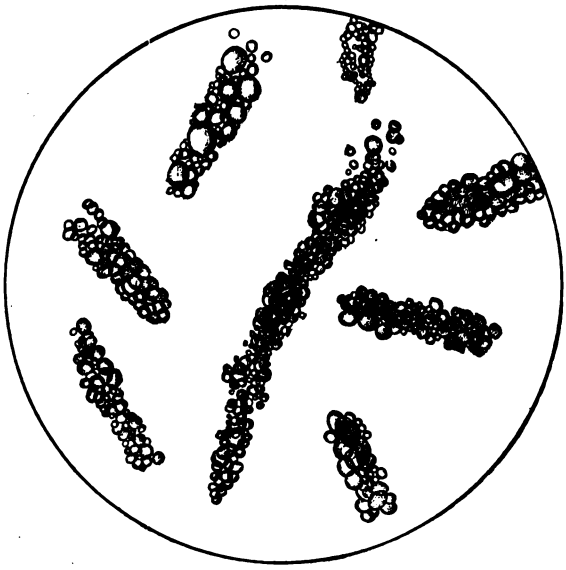


FIG. 112.—FATTY CASTS. (After *Peyer*.)

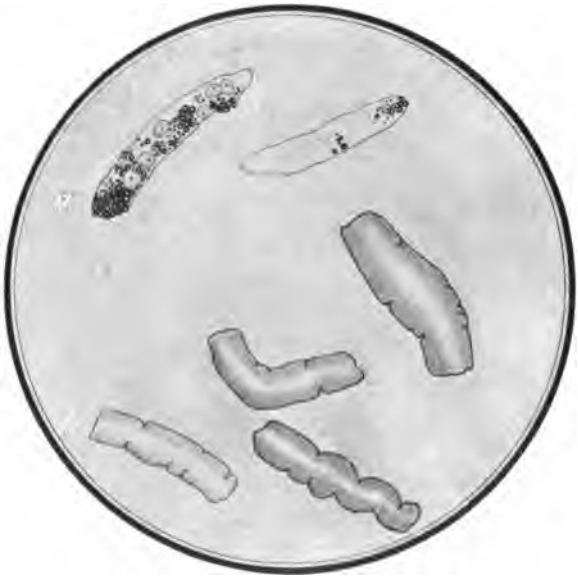


FIG. 113.—FATTY AND WAXY CASTS.
a, Fatty casts; *b*, waxy casts.

(*f*) *Waxy Casts.*—These casts possess a basic substance similar to that which enters into the foundation of the hyaline form of cast. In common with the hyaline type they are colorless, refractive bodies, but differ from this form of cast in being, in general, of greater length and diameter and possessing sharper outlines and a light yellow color (Fig. 113, p. 352). Such casts occur in several forms of nephritis, but do not appear to characterize any particular type of the disorder except *amyloid disease*, in which they are rather common.

(*g*) *Pus Casts.*—Casts whose surface is covered with pus cells or leucocytes are termed *pus casts* (Fig. 111, p. 351). They are frequently mistaken for epithelial casts. The differentiation between these two types is made very simple, however, by treating the cast with acetic acid which causes the nuclei of the leucocytes to become plainly visible. The true pus cast is quite rare and indicates renal suppuration.

FIG. 114.—CYLINDROIDS. (After *Peyer.*)

Cylindroids.—These formations may occur in normal or pathological urine and have no particular clinical significance. They are frequently mistaken for true casts, especially the hyaline type, but they are ordinarily *flat* in structure with a rather smaller diameter than casts, may possess forked or branching ends, and are not composed of homogeneous material as are the hyaline casts. Such "false casts" may become coated with urates, in which event they appear

granular in structure. The basic substance of cylindroids is often the nucleoprotein of the urine (see Fig. 114, page 353).

Erythrocytes.—These form elements are present in the urinary sediment in various diseases. They may appear as the normal biconcave, yellow erythrocyte (Plate IV, opposite page 180) or may exhibit certain modifications in form, such as the crenated type (Fig. 115, below) which is often seen in concentrated urine. Under different conditions they may become swollen sufficiently to entirely erase the biconcave appearance and may even occur in the form of colorless

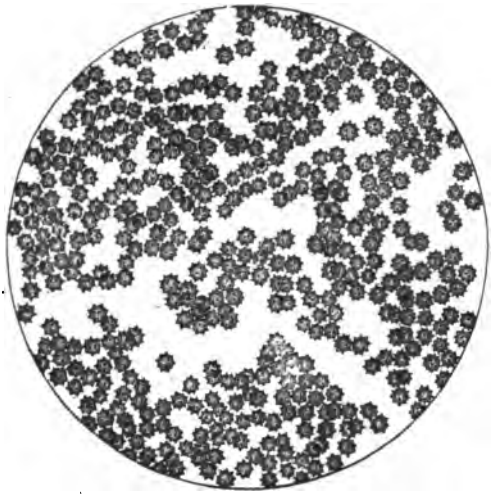


FIG. 115.—CRENATED ERYTHROCYTES.

spheres having a smaller diameter than the original disc-shaped corpuscles. Erythrocytes are found in urinary sediment in hemorrhage of the kidney or of the urinary tract, in traumatic hemorrhage, hemorrhage from congestion, and in hemorrhagic diathesis.

Spermatozoa.—Spermatozoa may be detected in the urinary sediment in diseases of the genital organs, as well as after coitus, nocturnal emissions, epileptic, and other convulsive attacks, and sometimes in severe febrile disorders, especially in typhoid fever. In form they consist of an oval body, to which is attached a long, delicate tail (Fig. 116, p. 355). Upon examination they may show motility or may be motionless.

Urethral Filaments.—These are peculiar thread-like bodies which are sometimes found in urinary sediment. They may occasionally be detected in normal urine and pathologically are found in the sediment in acute and chronic gonorrhœa and in urethrorrhœa.

The ground-substance of these urethral filaments is, in part at least, similar to that of the cylindroids (see page 353). The urine first voided in the morning is best adapted for the examination for filaments. These filaments may ordinarily be removed by a pipette since they are generally macroscopic.

Tissue Debris.—Masses of cells or fragments of tissue are frequently found in the urinary sediment. They may be found in the sediment in tubercular affections of the kidney and urinary tract or

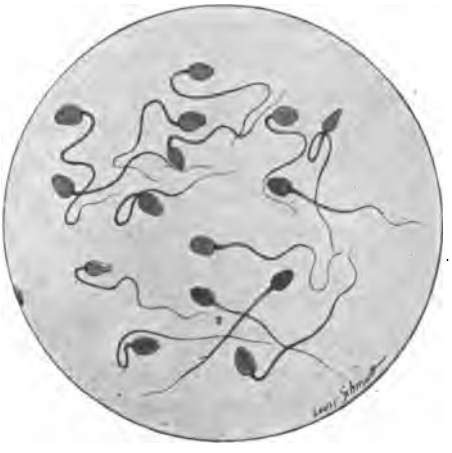


FIG. 116.—HUMAN SPERMATOZOA.

in tumors of these organs. Ordinarily it is necessary to make a histological examination of such tissue fragments before coming to a final decision as to their origin.

Animal Parasites.—The cysts, hooklets, and membrane shreds of *echinococci* are sometimes found in the urinary sediments. Other animal organisms which are more rarely met with in the urine are embryos of the *Filaria sanguinis* and eggs of the *Distoma hæmatobium* and *Ascarides*. Animal parasites in general occur most frequently in the urine in tropical countries.

Micro-organisms.—Bacteria as well as yeasts and moulds are frequently detected in the urine. Both the pathogenic and non-pathogenic forms of bacteria may occur. The non-pathogenic forms most frequently observed are *micrococcus ureæ*, *bacillus ureæ*, and *staphylococcus ureæ liquefaciens*. Of the pathogenic forms many have been observed, *e. g.*, *Bacterium Coli*, *typhoid bacillus*, *tubercle bacillus*, *gonococcus*, *bacillus pyocyaneus*, and *proteus vulgaris*. Yeast and moulds are most frequently met with in diabetic urine.

Fibrin.—Following hæmaturia, fibrin clots are occasionally observed in the urinary sediment. They are generally of a semi-gelatinous consistency and of a very light color, and when examined under the microscope they are seen to be composed of bundles of highly refractive fibers which run parallel.

Foreign Substances Due to Contamination.—Such foreign substances as fibers of silk, linen, or wool; starch granules, hair, fat, and sputum, as well as muscle fibers, vegetable cells, and food particles are often found in the urine. Care should be taken that these foreign substances are not mistaken for any of the true sedimentary constituents already mentioned.

CHAPTER XXI.

URINE: CALCULI.

URINARY *calculi*, also called *concretions*, or *concrements* are solid masses of urinary sediment formed in some part of the urinary tract. They vary in shape and size according to their location, the smaller calculi, termed *sand* or *gravel*, in general arising from the kidney or the pelvic portion of the kidney, whereas the large calculi are ordinarily formed in the bladder. There are two general classes of calculi as regards composition, *i. e.*, *simple* and *compound*. The simple form is made up of but a single constituent, whereas the compound type contains two or more individual constituents. The structural plan of most calculi consists of an arrangement of concentric rings about a central nucleus, the number of rings frequently being dependent upon the number of individual constituents which enter into the structure of the calculus. In case two or more calculi unite to form a single calculus the resultant body will obviously contain as many nuclei as there were individual calculi concerned in its construction. Under certain conditions the growth of a calculus will be principally in only one direction, thus preventing the nucleus from maintaining a central location. The qualitative composition of urinary calculi is dependent, in great part, upon the reaction of the urine, *e. g.*, if the reaction of the urine is acid the calculi present will be composed, in great part at least, of substances that are capable of depositing in acid urine.

According to Ultzmann, out of 545 cases of urinary calculus, uric acid and urates formed the nucleus in about 81 per cent of the cases; earthy phosphates in about 9 per cent; calcium oxalate in about 6 per cent; cystine in something over 1 per cent, while in about 3 per cent of the cases some foreign body comprised the nucleus.

In the chemical examination of urinary calculi the most valuable data are obtained by subjecting each of the concentric layers of the calculus to a separate analysis. Material for examination may be conveniently obtained by sawing the calculus carefully through the nucleus, then separating the various layers or by scraping off from each layer (without separating the layers) enough powder to conduct the examination as outlined in the scheme (see page 359).

Varieties of Calculus.

Uric Acid and Urate Calculi.—Uric acid and urates constitute the nuclei of a large proportion (81 per cent) of urinary concretions. Such stones are always colored, the tint varying from a pale yellow to a brownish-red. The surface of such calculi is generally smooth but it may be rough and uneven.

Phosphatic Calculi.—Ordinarily these concretions consist principally of "triple phosphate" and other phosphates of the alkaline earths, with very frequent admixtures of urates and oxalates. The surface of such calculi is generally rough but may occasionally be rather smooth. The calculi are somewhat variable in color, exhibiting gray, white, or yellow tints under different conditions. When composed of earthy phosphates the calculi are characterized by their friability.

Calcium Oxalate Calculi.—This is the hardest form of calculus to deal with, and is rather difficult to crush. They ordinarily occur in two general forms, *i. e.*, the small, smooth concretion which is characterized as the *hemp-seed calculus* and the medium-sized or large stone possessing an extremely uneven surface which is generally classed as a *mulberry calculus*. This roughened surface of the latter form of calculus is due, in many instances, to protruding calcium oxalate crystals of the octahedral type.

Calcium Carbonate Calculi.—Calcium carbonate concretions are quite common in herbivorous animals, but of exceedingly rare occurrence in man. They are generally small, white, or grayish calculi, spherical in form and possess a hard, smooth surface.

Cystine Calculi.—The cystine calculus is a rare variety of calculus. Ordinarily they occur as small, smooth, oval, or cylindrical concretions which are white or yellow in color and of a rather soft consistency.

Xanthine Calculi.—This form of calculus is somewhat more rare than the cystine type. The color may vary from white to brownish-yellow. Very often uric acid and urates are associated with xanthine in this type of calculus. Upon rubbing a xanthine calculus it has the property of assuming a wax-like appearance.

Urostealith Calculi.—This form of calculus is extremely rare. Such concretions are composed principally of fat and fatty acid. When moist they are soft and elastic, but when dried they become brittle. Urostealiths are generally light in color.

Fibrin Calculi.—Fibrin calculi are produced in the process of

On Heating the Powder on Platinum Foil, It				
Does burn				
Without flame		With flame		
The powder gives the murexide test	The powder when treated with KOH gives	No noticeable ammonia reaction	Strong ammonia reaction	
Does not give murexide test. The dried yellow residue becomes orange with alkali, beautiful red on warming		Flame pale blue, burns a short time. Peculiar sharp odor. The powder dissolves in ammonia, and six-sided plates separate on the spontaneous evaporation of the ammonia		Uric acid.
				Ammonium urate.
		Flame yellow, pale, continuous. Odor of resin or shellac on burning. Powder soluble in alcohol and ether		Xanthine.
				Cystine.
		Flame yellow, continuous. Odor of burnt feathers. Insoluble in alcohol and ether. Soluble in potassium hydroxide with heat. Precipitated herefrom by acetic acid and generation of hydrogen sulphide		Urostealith.
				Fibrin.
Does not burn				
The powder when treated with HCl				
Does not effervesce		Effervesces		Calcium carbonate.
The gently-heated powder with HCl		Effervesces		Calcium oxalate.
	The powder when moistened with a little KOH			
	No ammonia or, at least, only traces of ammonia. Powder dissolves in acetic acid or HCl. This solution is precipitated by ammonia (amorphous)			Bono-earth (magnesium and calcium phosphate).
	Abundant ammonia. The powder dissolves in acetic acid or HCl. This solution gives a crystalline precipitate with ammonia			"Triple phosphate" (mixed with unknown amount of earthy phosphate).

blood coagulation within the urinary tract. They frequently occur as nuclei of other forms of calculus. They are rarely found.

Cholesterol Calculi.—An extremely rare form of calculus somewhat resembling the cystine type.

Indigo Calculi.—Indigo calculi are extremely rare, only two cases having been reported. One of these indigo calculi is on exhibition in the museum of Jefferson Medical College of Philadelphia.

The scheme, proposed by Heller and given on page 359, will be found of much assistance in the chemical examination of urinary calculi.

CHAPTER XXII.

URINE: QUANTITATIVE ANALYSIS.

I. Protein.

1. **Scherer's Coagulation Method.**—The content of *coagulable* protein may be accurately determined as follows: Place 50 c.c. of urine in a small beaker and raise the temperature of the fluid to about 40° C. upon a water-bath. Add dilute acetic acid, drop by drop, to the warm urine, to precipitate the protein which will separate in a flocculent form. Care should be taken not to add too much acid; ordinarily less than twenty drops is sufficient. The temperature of the water in the water-bath should now be raised to the boiling-point and maintained there for a few minutes in order to insure the complete coagulation of the protein present. Now filter the urine[1] through a previously *washed*, *dried*, and *weighed* filter paper, wash the precipitated protein, in turn, with hot water, 95 per cent alcohol, and with ether, and dry the paper and precipitate, to constant weight, in an air-bath at 110° C. Subtract the weight of the filter paper from the combined weight of the paper and precipitate and calculate the percentage of protein in the urine specimen.

Calculation.—To determine the percentage of protein present in the urine under examination, multiply the weight of the precipitate, expressed in grams, by 2.

2. **Esbach's Method.**—This method depends upon the precipitation of protein by Esbach's reagent[2] and the apparatus used in the estimation is Esbach's albuminometer (Fig. 117, p. 362). In making a determination fill the albuminometer to the point U with urine, then introduce the reagent until the point R is reached. Now stopper the tube, invert it slowly several times in order to insure the thorough mixing of the fluids, and stand the tube aside for 24 hours. Creatinine, resin acids, etc., are precipitated in this method, and for this and

[1] If it is desired the precipitate may be filtered off on an *unweighed* paper, and its nitrogen content determined by the Kjeldahl method (see p. 375). In order to arrive at correct figures for the protein content it is then simply necessary to multiply the total nitrogen content by 6.25 (see p. 407). Correction should be made for the nitrogen content of the filter paper used unless this factor is negligible.

[2] Esbach's reagent is prepared by dissolving 10 grams of picric acid and 20 grams of citric acid in 1 liter of water.

other reasons it is not as accurate as the coagulation method. It is, however, extensively used clinically.

Calculation.—The graduations on the albuminometer indicate *grams of protein per liter of urine.* Thus, if the protein precipitate is level with the figure 3 of the graduated scale, this denotes that the urine examined contains 3 grams of protein to the liter. To express the amount of protein in *per cent* simply move the decimal point *one* place to the left. In the case under consideration the urine contains 0.3 per cent protein.

FIG. 117.—ESBACH'S ALBUMINOMETER. (*Ogden.*)

3. **Kwilecki's Modification of Esbach's Method.**[1]—Add 10 drops of a 10 per cent solution of $FeCl_3$ to the acid urine before introducing the Esbach's reagent. Warm the tube and contents in a water-bath at 72° C. for 5–6 minutes and make the reading.

II. Dextrose.

1. **Fehling's Method.**—Place 10 c.c. of the urine under examination in a 100 c.c. volumetric flask and make the volume up to 100 c.c. with distilled water. Thoroughly mix this diluted urine by pouring it into a beaker and stirring with a glass rod, then transfer a portion of it to a burette which is properly supported in a clamp.

Now place 10 c.c. of Fehling's solution[2] in a small beaker, dilute it with approximately 40 c.c. of distilled water, heat to boiling, and observe whether decomposition of the Fehling's solution itself has occurred as indicated by the production of a turbidity. If such turbidity is produced the Fehling's solution is unfit for use. Clamp the burette containing the dilute urine immediately over the beaker and carefully allow from 0.5 to 1 c.c. of the diluted urine to flow into the *boiling* Fehling's solution. Bring the solution to the boiling-point after each addition of urine and continue running in the urine from the burette, 0.5–1 c.c. at a time, as indicated, until the Fehling's solution is *completely reduced, i. e.*, until all the cupric oxide in solution has been precipitated as cuprous oxide. This point will be indicated

[1] Kwilecki: *Münch. Med. Woch.*, LVI, p. 1330.

[2] Directions for the preparation of Fehling's solution are given in a note at the bottom of page 27.

by the *absolute disappearance of all blue color.* When this end-point is reached note the number of cubic centimeters of diluted urine used in the process and calculate the percentage of dextrose present, in the sample of urine analyzed, according to the method given below.

This is a very satisfactory method, the main objection to its use being the uncertainty attending the determination of the end-reaction, *i. e.*, the difficulty with which the exact point where the blue color *finally disappears* is noted. Several means of accurately fixing this point have been suggested, but they are practically all open to objection. As good a "check" as any, perhaps, is to filter a few drops of the solution, through a double paper, after the blue color has *apparently* disappeared, acidify the filtrate with acetic acid and add potassium ferrocyanide. If the copper of the Fehling's solution has been completely reduced, there will be no color reaction, whereas the production of a brown color indicates the presence of *unreduced* copper. Harrison has recently suggested the following procedure to determine the exact end-point: To about 1 c.c. of a starch iodide solution[1] in a test-tube add 2–3 drops of acetic acid and introduce into the acidified mixture 1–2 drops of the solution to be tested. *Unreduced copper* will be indicated by the production of a *purplish-red* or *blue* color due to the liberation of iodine.

It is ordinarily customary to make at least three determinations by Fehling's method before coming to a final conclusion regarding the sugar content of the urine under examination.

Calculation.—Ten c.c. of Fehling's solution is completely reduced by 0.05 gram of dextrose.[2] If y represents the number of cubic centimeters of *undiluted* urine (obtained by dividing the burette reading by 10) necessary to reduce the 10 c.c. of Fehling's solution, we have the following proportion:

$$y : 0.05 :: 100 : x \text{ (percentage of dextrose).}$$

2. **Benedict's Method.**—To 30 c.c. of Benedict's solution[3] in

[1] The starch-iodide solution may be prepared as follows: Mix 0.1 gram of starch with *cold* water in a mortar and pour the suspended starch granules into 75–100 c.c. of *boiling* water, stirring continuously. Cool the starch paste, add 20–25 grams of potassium iodide and dilute the mixture to 250 c.c. This solution deteriorates upon standing, and therefore must be freshly prepared as needed.

[2] The values for certain other sugars are as follows:

Lactose	0.0676 gram.
Maltose	0.074 gram.
Invert sugar	0.0475 gram.

[3] Benedict's solution used in the quantitative determination of sugar consists of *three* separate solutions. The *cupric sulphate solution* and the *alkaline tartrate solution* are the same as those already described in connection with Benedict's qualitative test, see p. 309. The third solution is made up as follows:

Potassium ferro-thiocyanate solution = 15 grams of potassium ferrocyanide, 62.5 grams

a small beaker add from 2.5 grams to 5 grams of anhydrous sodium carbonate[1] and heat the mixture to boiling over a wire gauze until the carbonate has been brought into solution.

Place the urine under examination in a burette and run it into the hot Benedict solution rather rapidly[2] until the formation of a heavy *chalk-white precipitate* is noted and the blue color of the solution lessens perceptibly in its intensity. From this point in the determination from 2 to 10 drops[3] of the urine should be run into the boiling Benedict solution at one time, boiling the solution vigorously for about 15 seconds after each addition. Complete reduction of the copper is indicated here as in Fehling's original method, by the *complete disappearance of all blue color*. The end-point here, however, is very sharply defined, contrary to the conditions in the older method.

To prevent the annoying bumping which often interferes with the titration, a medium-sized piece of washed absorbent cotton[4] may be introduced into the solution. This cotton may be stirred about through the solution as the titration proceeds and the bumping thus eliminated.

Calculation.—Thirty cubic centimeters of Benedict's solution is completely reduced by 0.073 gram of *dextrose*. If y represents the number of cubic centimeters of urine necessary to reduce the 30 c.c. of the solution we have the following proportion:

$$y : 0.073 :: 100 : x \text{ (percentage of dextrose).}$$

3. **Purdy's Method.**—Purdy's solution[5] is a modification of Fehling's solution and is said to possess greater stability than the

of potassium thiocyanate and 50 grams of anhydrous sodium carbonate dissolved in water and made up to 500 c.c.

These *three* solutions should be preserved *separately* in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

[1] The amount added depends upon the dilution to which the solution is to be subjected in titration. For this reason the maximum amount of sodium carbonate should be added when titrating urines containing a very low percentage of sugar.

[2] Not rapidly enough, however, to interfere in any marked degree with the continuous vigorous boiling of the solution.

[3] The exact amount to run in depends upon the intensity of the remaining blue color, as well as upon the sugar content of the urine. The 10 drops should be added at one time only when urines containing a very low percentage of sugar are under examination.

[4] Glass wool may be substituted if desired.

[5] Purdy's solution has the following composition:

Cupric sulphate	4.752	grams.
Potassium hydroxide	23.5	grams.
Ammonia (U. S. P., sp. gr. 0.9)	350.0	c.c.
Glycerol	38.0	c.c.

Distilled water, to make total volume 1 liter.

In preparing the solution bring the cupric sulphate and potassium hydroxide into solution in separate vessels, mix the two solutions, cool the mixture, and add the ammonia and glycerol. After this has been done the total volume should be made up to 1 liter with distilled water.

Thirty-five cubic centimeters of Purdy's solution is exactly reduced by 0.02 gram of dextrose.

latter. One of the most satisfactory points about the method as suggested by Purdy is the ease with which the exact end-reaction may be determined. In determining the percentage of dextrose by this method proceed as follows: Place 35 c.c. of Purdy's solution in a 200 c.c. Erlenmeyer flask and dilute the fluid with approximately two volumes of distilled water. Fit a cork, provided with two perforations, to the neck of the flask and through one perforation introduce the tip of a burette and through the second perforation introduce a tube bent at right angles in such a manner as to allow the steam to escape and keep the fumes of ammonia away from the face of the operator as completely as possible.[1] Now bring the solution to the boiling-point and add the urine, drop by drop, until the intensity of the *blue color begins to diminish*. When this point is reached add the urine somewhat *more slowly* until the blue color is entirely dissipated and an *absolutely decolorized* solution remains. Take the burette reading and calculate the percentage of dextrose in the urine examined according to the method given below.

Care should be taken not to boil the solution for too long a period, since, under these conditions, sufficient ammonia might be lost to allow the cuprous hydroxide to precipitate.

Some investigators consider it to be advisable to dilute the urine before applying the above manipulation, but ordinarily this is not necessary unless the urine has a high content of dextrose (5 per cent or over). In this event the urine may be diluted with 2–3 volumes of water and the proper correction made in the calculation.

Calculation.—Thirty-five c.c. of Purdy's solution is completely reduced by 0.02 gram of dextrose. If y represents the number of cubic centimeters of *undiluted* urine necessary to reduce 35 c.c. of Purdy's solution, we have the following proportion:

$$y : 0.02 :: 100 : x \text{ (percentage of dextrose).}$$

4. **Fermentation Method.**—This method consists in the measurement of the volume of carbon dioxide evolved when the dextrose of the urine undergoes fermentation with yeast. None of the various methods whose manipulation is based upon this principle is *absolutely* accurate. The method in which Einhorn's saccharometer (Fig. 2, page 31) is the apparatus employed is perhaps as satisfactory as any for clinical pur-

[1] This side tube may also be equipped with a simple air-valve, thus insuring the exclusion of air and thereby contributing to the accuracy of the determination, inasmuch as the cuprous salts would be reoxidized upon coming in contact with the air. If one is careful to maintain the solution continuously at the boiling-point throughout the entire process, however, there is no opportunity for air to enter and therefore no need of an air-valve.

poses. The procedure is as follows: Place about 15 c.c. of urine in a mortar, add about 1 gram of yeast (1/16 of the ordinary cake of compressed yeast) and carefully crush the latter by means of a pestle. Transfer the mixture to the saccharometer, being careful to note that the graduated tube is *completely* filled and that no air bubbles gather at the top. Allow the apparatus to stand in a warm place (30° C.) for 12 hours and observe the percentage of dextrose as indicated by the graduated scale of the instrument. Both the percentage of dextrose and the number of cubic centimeters of carbon dioxide are indicated by the graduations on the side of the saccharometer tube.

5. **Polariscopic Examination.**—Before subjecting urine to a polariscopic examination the slightly acid fluid should be decolorized as thoroughly as possible by the addition of a little plumbic acetate. The urine should be well stirred and then filtered through a filter paper which has not been previously moistened. In this way a perfectly *clear* and almost colorless liquid is obtained.

In determining dextrose by means of the polariscope it should be borne in mind that this carbohydrate is often accompanied by other optically active substances, such as proteins, lævulose, β-oxybutyric acid, and conjugate glycuronates which may introduce an error into the polariscopic reading; the method is, however, sufficiently accurate for practical purposes.

For directions as to the manipulation of the polariscope see page 31.

III. Uric Acid.

1. **Folin-Shaffer Method.**—Introduce 100 c.c.[1] of urine into a beaker, add 25 c.c. of the Folin-Shaffer reagent[2] and allow the mixture to stand,[3] without further stirring, until the precipitate has settled (5–10 minutes). Filter, transfer 100 c.c. of the filtrate to a 200 c.c. beaker or Erlenmeyer flask, add 5 c.c. of concentrated ammonium hydroxide and allow the mixture to stand for 24 hours. Transfer the precipitated ammonium urate quantitatively to a filter paper,[4] using 10 per cent ammonium sulphate to remove the final traces of the urate from the beaker. Wash the precipitate *approximately* free from chlorides by

[1] It is preferable to use more than 100 c.c. of urine if the fluid has a specific gravity less than 1.020.

[2] The Folin-Shaffer reagent consists of 500 grams of ammonium sulphate, 5 grams of uranium acetate and 60 c.c. of 10 per cent acetic acid in 650 c.c. of distilled water.

[3] The mixture should not be allowed to stand for too long a time at this point, since uric acid may be lost through precipitation.

[4] The Schleicher and Schüll *hardened* papers or the Baker and Adamson *washed, ashless* variety are very satisfactory for this purpose.

means of 10 per cent ammonium sulphate solution,[1] remove the paper from the funnel, open it, and by means of *hot* water rinse the precipitate back into the beaker in which the urate was originally precipitated. The volume of fluid at this point should be about 100 c.c. Cool the solution to room temperature, add 15 c.c. of concentrated sulphuric acid and titrate at once with N/20 potassium permanganate, $K_2Mn_2O_8$, solution. The first tinge of pink color which extends throughout the fluid after the addition of *two drops* of the permanganate solution, while stirring with a glass rod, should be taken as the end-reaction. Take the burette reading and compute the percentage of uric acid present in the urine under examination.

Calculation.—Each cubic centimeter of N/20 potassium permanganate solution is equivalent to 3.75 milligrams (0.00375 gram) of uric acid. The 100 c.c. from which the ammonium urate was precipitated is equivalent to only four-fifths of the 100 c.c. of urine originally taken, therefore we must take five-fourths of the burette reading in order to ascertain the number of cubic centimeters of the permanganate solution required to titrate 100 c.c. of the *original urine* to the correct end-point. If y represents the number of cubic centimeters of the permanganate solution required, we may make the following calculation:

$$y \times 0.00375 = \text{weight of uric acid in 100 c.c. of urine.}$$

Because of the solubility of the ammonium urate a correction of 3 milligrams should be added to the final result.

Calculate the quantity of uric acid in the twenty-four-hour urine specimen.

2. **Heintz Method.**—This is a very simple method and was the first one in general use for the quantitative determination of uric acid. It is believed to be somewhat less accurate than the method just described. The procedure is as follows: Place 100 c.c. of filtered urine in a beaker, add 5 c.c. of concentrated hydrochloric acid, stir the fluid thoroughly, and stand it away in a cool place for 24 hours. Filter off the uric acid crystals upon a washed, dried and *weighed* filter paper and wash them with *cold* distilled water, a few cubic centimeters at a time until the chlorides are removed. Now wash, in turn, with alcohol and with ether and finally dry the paper and crystals to constant weight at 110° C. In the process of washing the uric acid free from chlorides an error is introduced, since every cubic centimeter of water so used dissolves 0.00004 gram of uric acid. For this reason a correc-

[1] This washing may be conveniently done by *decantation* if desired, thus retaining the major portion of the precipitate in the beaker or flask.

tion is necessary. It has been suggested that the pigment of the crystals is equivalent in weight to the amount of uric acid dissolved by the first 30 c.c. of water, and this factor should be taken into account in the computation of the percentage of uric acid.

Calculation.—Since 100 c.c. of urine was used the *corrected* weight of the uric acid crystals, in grams, will express the *percentage* of uric acid present.

3. **Krüger and Schmidt's Method.**—This method serves for the detection of both uric acid and the purine bases. The principle involved is the precipitation of both the uric acid and the purine bases in combination with copper oxide and the subsequent decomposition of this precipitate by means of sodium sulphide. The uric acid is then precipitated by means of hydrochloric acid and the purine bases are separated from the filtrate in the form of their copper or silver compounds. The nitrogen content of the precipitates of uric acid and purine bases is then determined by means of the Kjeldahl method (see p. 375) and the corresponding values for uric acid and purine bases calculated. The method is as follows: To 400 c.c. of albumin-free urine[1] in a liter flask,[2] add 24 grams of sodium acetate, 40 c.c. of a solution of sodium bisulphite[3] and heat the mixture to boiling. Add 40–80 c.c.[4] of a 10 per cent solution of cupric sulphate and maintain the temperature of the mixture at the boiling-point for at least three minutes. Filter off the flocculent precipitate, wash it with hot water until the wash water is colorless, and return the washed precipitate to the flask by puncturing the tip of the filter paper and washing the precipitate through by means of hot water. Add water until the volume in the flask is approximately 200 c.c., heat the mixture to boiling, and decompose the precipitate of copper oxide by the addition of 30 c.c. of sodium sulphide solution.[5] After decomposition is complete, the mixture should be acidified with acetic acid and heated to boiling until the separating sulphur collects in a mass. Filter the hot fluid by means of a filter pump, wash with hot water, add 10 c.c. of 10 per cent hydro-

[1] If albumin is present, the urine should be heated to boiling, acidified with acetic acid and filtered.

[2] The total volume of urine for the twenty-four hours should be sufficiently diluted with water to make the total volume of the solution 1600–2000 c.c.

[3] A solution containing 50 grams of Kahlbaum's commercial sodium bisulphite in 100 c.c. of water.

[4] The exact amount depending upon the content of the purine bases.

[5] This is made by saturating a 1 per cent solution of sodium hydroxide with hydrogen sulphide gas and adding an equal volume of 1 per cent. sodium hydroxide.

Ordinarily the addition of 30 c.c. of this solution is sufficient, but the presence of an excess of sulphide should be *proven* by adding a drop of lead acetate to a drop of the solution. Under these conditions a dark brown color will show the presence of an excess of sodium sulphide

chloric acid and evaporate the filtrate in a porcelain dish until the total volume has been reduced to about ten cubic centimeters. Permit this residue to stand about two hours to allow for the separation of the uric acid, leaving the purine bases in solution. Filter off the precipitate of uric acid, using a small filter paper, and wash the uric acid, with water made acid with sulphuric acid, until the total volume of the original filtrate and the wash water aggregates 75 c.c. Determine the nitrogen content of the precipitate by means of the Kjeldahl method (see p. 375) and calculate the uric acid equivalent.

Calculation.—In calculating the uric acid value from the total nitrogen simply multiply the latter by *three* and add 0.0035 to the product as a correction for the uric acid remaining in solution in the 75 c.c.

FIG. 118.—MARSHALL'S UREA APPARATUS. (*Tyson.*)

a, Bulbed measuring tube; *b*, saucer-shaped vessel; *c*, graduated pipette; *d*, funnel-tube.

IV. Urea.

1. **Knop-Hüfner Hypobromite Method (using Marshall's Urea Apparatus).**—Place the thumb over the side opening of the bulbed-tube of the apparatus (Fig. 118) and carefully fill the tube with sodium hypobromite solution.[1] Close the opening in the end of the tube with a rubber stopper, incline the tube to allow air-bubbles to escape, and finally invert the tube and fix the stoppered end in the saucer-shaped vessel. By means of the graduated pipette *rapidly* introduce 1 c.c. of urine[2] into the hypobromite solution through the side opening of the bulbed-tube. Withdraw the pipette immediately after the urine has been introduced. When the decomposition of the urea is completed (10–20 minutes) gently tap the bulbed-tube with the finger in order to dislodge any

[1] The ingredients of the sodium hypobromite solution should be prepared in the form of *two* separate solutions. When needed for use mix one volume of solution *a*, one volume of solution *b*, and 3 volumes of water.

(*a*) Dissolve 125 grams of sodium bromide in water, add 125 grams of bromine and make the total volume of the solution 1 liter.

(*b*) A solution of sodium hydroxide having a specific gravity of 1.250. This is approximately a 22.5 per cent solution.

Preserve both solutions in rubber-stoppered bottles.

[2] Ordinarily 1 c.c. of urine is sufficient; more may be used, however, if its content of urea is *very low*.

gas bubbles which may have collected on the inner surface of the glass. The atmospheric pressure should now be equalized by attaching the funnel-tube to the bulbed-tube at the side opening and introducing hypobromite solution into it until the columns of liquid in the two tubes are uniform in height. The graduated scale of the bulbed-tube should now be read in order to determine the number of cubic centimeters of nitrogen gas evolved. By means of the appended formula the *weight* of the urea present in the urine under examination may be computed.

Calculation.[1]—By properly substituting in the following formula the *weight* of urea, in grams, contained in the volume of urine decomposed (1 c.c. or more) may readily be determined:

$$w = \frac{v(p - T)}{354.5 \times 760(1 + 0.003665t)}$$

w = weight of urea, in grams.

v = observed volume of nitrogen expressed in cubic centimeters.

p = barometric pressure expressed in mm. of mercury.

T = tension of aqueous vapor[2] for temperature t.

t = temperature (centigrade).

If we wish to calculate the *percentage* of urea we may do so by means of the following proportion in which y represents the volume of urine used and w denotes the weight of the urea contained in the volume y:

$$y : w :: 100 : x \text{ (percentage of urea).}$$

Sodium hypobromite solution may also be employed for the determination of urea in the apparatus devised by Hüfner which is pictured in Fig. 119, page 371.

2. **Knop-Hüfner Hypobromite Method (Using the Doremus-Hinds Ureometer).**—In common with the method already described, this method depends upon the measurement of the volume of nitrogen gas liberated when the urea of the urine is decomposed by means of sodium hypobromite solution. The Doremus-Hinds ureometer (Fig.

[1] 0.003665 = coefficient of expansion of gases for 1° C. 354.5 = number of c.c. of nitrogen gas evolved from 1 gram of urea.

[2] The values of T for the temperatures ordinarily met with are given in the following table:

Temp.	Tension in mm.	Temp.	Tension in mm.
15° C.	12.677	21° C.	18.505
16° C.	13.519	22° C.	19.675
17° C.	14.009	23° C.	20.909
18° C.	15.351	24° C.	22.211
19° C.	16.345	25° C.	23.582
20° C.	17.396		

120, p. 372), is one of the simplest and cheapest forms of apparatus in general use for the determination of urea by the hypobromite process. In using this apparatus proceed as follows: Fill the side tube B and the lumen of the stopcock C with the urine under examination. Carefully wash out tube A with water and introduce into it sodium hypobromite solution,[1] being careful to fill the bulb sufficiently full to prevent the entrance of air into the graduated portion. Now allow 1 c.c. of urine[2] to flow from tube B into tube A and after the evolution of gas bubbles has ceased (10–20 minutes) take the reading of the graduated scale on tube A.

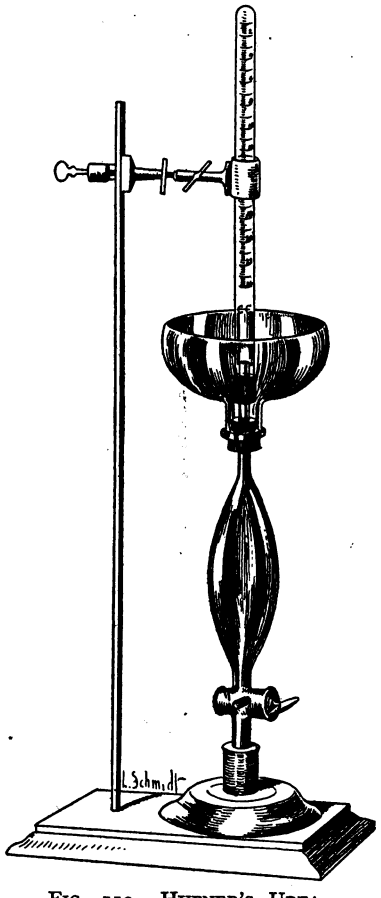


FIG. 119.—HUFNER'S UREA APPARATUS.

In common with all other methods which are based upon the decomposition of urea by means of hypobromite solution, this method is not absolutely correct. It is, however, sufficiently accurate for ordinary clinical purposes.

Calculation.—Observe the reading on the graduated scale of tube A. This tube is so graduated as to represent the weight of urea, in grams, per cubic centimeter of urine. If we wish to compute the *percentage* of urea present this may be done very readily by simply moving the decimal point *two places to the right; e. g.*, if the reading is 0.02 gram the urine contains 2 per cent of urea.

3. **Folin's Method.**—This is one of the most accurate methods yet devised for the determination of urea in the urine. The procedure is as follows: Place 5 c.c. of urine in a 200 c.c. Erlenmeyer flask and add to it 5 c.c. of concentrated hydrochloric acid, 20 grams of crystallized magnesium chloride, a piece of paraffin the size of a hazel nut, and 2–3 drops of a 1 per cent aqueous solution of "alizarin red." Insert a Folin safety

[1] For directions as to the preparation of this solution see page 369.

[2] If the content of urea in the urine under examination is large, the urine may be diluted with water before determining the urea. If this is done it must of course be taken into consideration in computing the content of urea.

tube (Fig 121, p. 373) into the neck of the flask and boil the mixture until each drop of reflow from the safety tube produces a very perceptible bump; the heat is then reduced somewhat and continued one and one-half hours. The contents of the flask must not remain alkaline, and to obviate this, at the first appearance of a reddish tinge in the contents of the flask *a few drops* of the acid distillate are shaken back into the flask. At the end of 1 1/2 hours the contents of the vessel are transferred to a 1-liter flask with about 700 c.c. of distilled water, about 20 c.c. of 10 per cent potassium hydroxide or sodium hydroxide solution is added and the mixture distilled into a known volume of N/10 sulphuric acid until the contents of the flask are nearly dry or until the distillate fails to give an alkaline reaction to litmus, showing the absence of ammonia. The time devoted to this process is ordinarily about an hour. Boil the distillate a few moments to free it from CO_2, then cool and titrate the mixture with N/10 sodium hydroxide, using "alizarin red" as indicator.

FIG. 120.—DOREMUS-HINDS UREOMETER.

A "check" experiment should always be made to determine the original ammonia content of the urine and of the magnesium chloride, if it is not absolutely pure, which of course should be subtracted from the total amount of ammonia as determined by the above process.

The Folin method is extremely accurate under all conditions *except when the urine contains sugar*. When this is the case the carbohydrate and the urea unite, upon being heated, and form a very stable combination. For this reason the Folin method is not suitable for use in the examination of such urines. The best method for use under such conditons is the combination Mörner-Sjöqvist-Folin method which is given below.

4. **Mörner-Sjögvist-Folin Method.**—As has already been stated in the last experiment, this method excels the Folin method in accuracy *only* in the determination of urea in the presence of carbohydrate bodies.

Briefly, the procedure is as follows:[1] Bring the major portion of 1.5 gram of powdered barium hydroxide into solution in 5 c.c. of urine in a small flask, and treat the mixture with 100 c.c. of an alcohol-ether solution, consisting of two volumes of 97 per cent alcohol and one volume of ether. Stopper the flask and allow it to stand 12–24 hours. Filter off the precipitate, wash it with the alcohol-ether mixture and remove the alcohol and ether from the filtrate by distillation, being careful to keep the temperature of the mixture below 50° C.[2] Treat the remaining fluid (about 25 c.c.) with 2 c.c. of hydrochloric acid (sp. gr. 1.124), transfer it carefully to a 200 c.c. flask, and evaporate the mixture to dryness on a water-bath. Now add 20 grams of crystallized magnesium chloride and 2 c.c. of concentrated hydrochloric acid to the residue, and after fitting the flask with a return cooler boil the mixture on a wire gauze over a small flame for two hours. Cool the solution, dilute to 750 c.c. or 1000 c.c. with water, render the mixture alkaline with potassium hydroxide or sodium hydroxide, distil off the ammonia and collect it in an acid solution of known strength. Boil the distillate to remove carbon dioxide, cool and titrate with an alkali of known strength. In this method, as well as in Folin's method (see p. 371), correction must be made for the ammonia originally present in the urine and in the magnesium chloride.

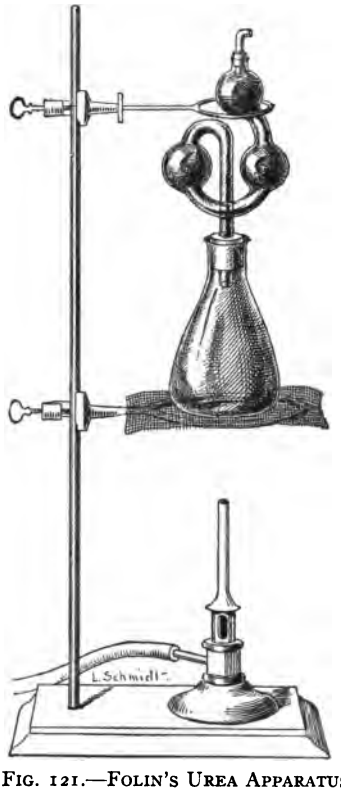


FIG. 121.—FOLIN'S UREA APPARATUS.

5. **Benedict's Method.**[3]—Five cubic centimeters of urine are introduced into a rather wide test-tube, about 3 grams of potassium bisulphate and 1–2 grams of zinc sulphate added, a small quantity of powdered pumice and a bit of paraffin are introduced and the mixture boiled almost to dryness either over a free flame or by immersion in a sul-

[1] The original description of the method may be found in an article by Mörner: *Skandinavisches Archiv für Physiologie*, 1903, XIV, p. 297.
[2] There is some decomposition of urea at 60° C.
[3] Private communication from Dr. S. R. Benedict.

phuric acid bath at about 130°. The tubes are then weighted (a screw clamp is convenient) and immersed for three-fourths of their length in a bath of sulphuric acid at a temperature of 160–163° for one hour. The residue in the tube is then dissolved in water and distilled as usual (see Kjeldahl Method, p. 375), boiling with sodium carbonate in place of hydroxide.

V. Ammonia.

1. **Folin's Method.**—Place 25 c.c. of urine in an aerometer cylinder, 30–40 cm. in height (Fig. 122, below), add about 1 gram of dry sodium carbonate and introduce some crude petroleum to prevent foaming.

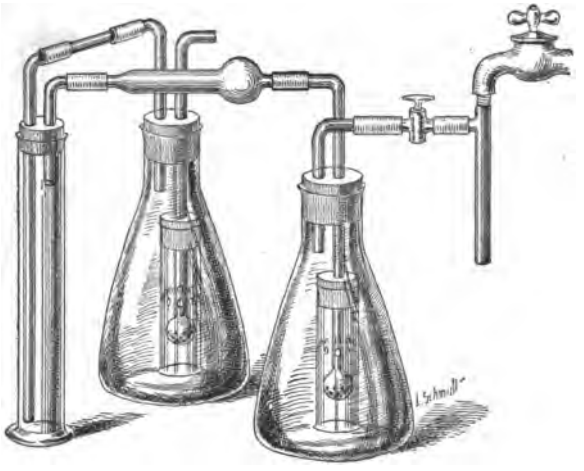


FIG. 122.—FOLIN'S AMMONIA APPARATUS.

Insert into the neck of the cylinder a rubber stopper provided with two perforations, into each of which passes a glass tube, one of which reaches below the surface of the liquid. The shorter tube (10 cm. in length) is connected with a calcium chloride tube filled with cotton, and this tube is in turn joined to a glass tube extending to the bottom of a 500 c.c. wide-mouthed flask which is intended to absorb the ammonia and for this purpose should contain 20 c.c. of N/10 sulphuric acid, 200 c.c. of distilled water and a few drops of an indicator ("alizarin red"). To insure the complete absorption of the ammonia the absorption flask is provided with a Folin improved absorption tube (Fig. 123, p. 375) which is very effective in causing the air passing from the cylinder to come into intimate contact with the acid in the absorption flask. In order to exclude any error due to the presence of ammonia in the air a

similar absorption apparatus to the one just described is attached to the other side of the aerometer cylinder, thus insuring the passage of *ammonia-free* air into the cylinder. With an ordinary filter pump and good water pressure the last trace of ammonia should be removed from the cylinder in about one and one-half hours.[1] The number of cubic centimeters of the N/10 sulphuric acid neutralized by the ammonia of the urine may be determined by direct titration with N/10 sodium hydroxide.

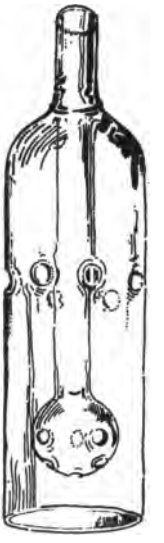


FIG. 123.—FOLIN IMPROVED ABSORPTION TUBE.

This is one of the most satisfactory methods yet devised for the determination of ammonia. Steele[2] has recently suggested a modification.

Calculation.—Subtract the number of cubic centimeters of N/10 sodium hydroxide used in the titration from the number of cubic centimeters of N/10 sulphuric acid taken. The remainder is the number of cubic centimeters of N/10 sulphuric acid *neutralized by the* NH_3 of the urine. 1 c.c. of N/10 sulphuric acid is equivalent to 0.0017 *gram of* NH_3. Therefore if y represents the volume of urine used in the determination and y' the number of cubic centimeters of N/10 sulphuric acid *neutralized by the* NH_3 *of the urine*, we have the following proportion:

$$y : 100 :: y' \times 0.0017 : x \text{ (percentage of } NH_3 \text{ in the urine examined).}$$

Calculate the quantity of NH_3 in the twenty-four-hour urine specimen.

VI. Nitrogen.

Kjeldahl Method.[3]—The principle of this method is the conversion of the various nitrogenous bodies of the urine into ammonium sulphate by boiling with concentrated sulphuric acid, the subsequent decomposition of the ammonium sulphate by means of a fixed alkali (NaOH) and the collection of the liberated ammonia in an acid of known strength. Finally, this partly neutralized acid solu-

[1] With any given filter pump a "check" test should be made with urine or better with a solution of an ammonium salt of known strength to determine how long the air current must be maintained to remove all the ammonia from 25 c.c. of the solution.

[2] Steele: Proc. Soc. Exp. Biol. and Med., 6, p. 127.

[3] There are numerous modifications of the original Kjeldahl method; the one described here, however, has given excellent satisfaction and is recommended for the determination of the nitrogen content of urine.

tion is titrated with an alkali of known strength and the nitrogen content of the urine under examination computed.

The procedure is as follows: Place 5 c.c. of urine in a 500 c.c. long-necked Jena glass Kjeldahl flask, add 20 c.c. of concentrated sulphuric acid and about 0.2 gram of cupric sulphate and boil the mixture for some time after it is colorless (about one hour.) Allow the flask to cool and dilute the contents with about 200 c.c. of ammonia-free water. Add a little more of a concentrated solution of NaOH than is necessary to neutralize the sulphuric acid[1] and introduce into the flask a little coarse pumice stone or a few pieces of granulated zinc,[2] to prevent bumping, and a small piece of paraffin to lessen the tendency to froth. By means of a safety-tube connect the flask with a condenser so arranged that the delivery-tube passes into a vessel containing a known volume (the volume used depending upon the nitrogen content of the urine) of N/10 sulphuric acid, using care that the end of the delivery-tube reaches beneath the surface of the fluid.[3] Mix the contents of the distillation flask very thoroughly by shaking and distil the mixture until its volume has diminished about one-half. Titrate the partly neutralized N/10 sulphuric acid solution by means of N/10 sodium hydroxide, using congo red as indicator, and calculate the content of nitrogen of the urine examined.

Calculation.—Subtract the number of cubic centimeters of N/10 sodium hydroxide used in the titration from the number of cubic centimeters of N/10 sulphuric acid taken. The remainder is equivalent to the number of cubic centimeters of N/10 sulphuric acid, *neutralized by the ammonia of the urine.* One c.c. of N/10 sulphuric acid is equivalent to 0.0014 *gram of nitrogen.* Therefore, if y represents the volume of urine used in the determination, and y' the number of cubic centimeters of N/10 sulphuric acid *neutralized by the ammonia of the urine*, we have the following proportion:

$y : 100 : : y' \times 0.0014 : x$ (percentage of nitrogen in the urine examined).

Calculate the quantity of nitrogen in the twenty-four-hour urine specimen.

VII. Hippuric Acid.

Dakin's Methods.[4]—*Preliminary Procedure.*—Place 150 c.c. (or more) of the urine under examination in a porcelain evaporating

[1] This concentrated sodium hydroxide solution should be prepared in quantity and "check" tests made to determine the volume of the solution necessary to neutralize the volume (20 c.c.) of concentrated sulphuric acid used.

[2] Powdered zinc may be substituted.

[3] This delivery-tube should be of large caliber in order to avoid the "sucking back" of the fluid.

[4] Private communication to the author from Dr. H. D. Dakin.

dish and evaporate *almost* to dryness upon a water-bath. Add about 1 gram of sodium dihydrogen phosphate, about 25 grams of gypsum ($CaSO_4$, $2H_2O$) and rub up with a pestle and stir with a spatula until a uniform mixture results. Dry the powder thus produced in a water-oven for about two hours, at the end of which period it should be rubbed up a second time, to remove lumps, and transferred to a Schleicher and Schüll "extraction shell" and extracted in a Soxhlet apparatus in the usual way (see p. 405). The extraction medium is ethyl acetate and the flask containing the acetate should be strongly heated over a *sand-bath*[1] for about two hours. The ethyl acetate extract is now transferred to a separatory funnel, and the original flask rinsed with sufficient fresh ethyl acetate to make the total volume in the separatory funnel[2] about 100 c.c. Wash the ethyl acetate solution *five times* with a saturated solution of sodium chloride, using 8 c.c. of the sodium chloride solution at each extraction, shaking vigorously and removing the sodium chloride extract in each case before adding fresh sodium chloride solution. The sodium chloride *removes the urea completely* and the hippuric acid is then determined in the urea-free solution by the following volumetric or gravimetric procedure:

1. *Volumetric Determination.*—Transfer the urea-free ethyl acetate solution, prepared as described above, to a Kjeldahl flask, add about 25 c.c. of water, a small piece of pumice stone to prevent bumping, attach a condenser and distil off the ethyl acetate[3] over a free flame. After practically all of the ethyl acetate has been distilled off, the nitrogen in the remaining solution should be determined by means of the Kjeldahl method (see p. 375).

The main source of error in this method is the fact that any nitrogen present in the form of *phenaceturic acid* or *indole acetic acid* is determined as hippuric acid nitrogen. The error from this source is, however, usually trifling.

Calculation.—Calculate as usual for nitrogen determinations, remembering that 1 *c.c. of* N/10 *sulphuric acid is equivalent to* 0.0179 *gram hippuric acid.*

2. *Gravimetric Determination.*—The urea-free ethyl acetate solution, contained in the separatory funnel, after washing with sodium chloride solution, as described under Preliminary Procedure, p. 376, is washed with 5 c.c. of distilled water to remove the major portion of

[1] A water-bath cannot be substituted inasmuch as the resultant extraction would be *too slow.*

[2] This ethyl acetate solution contains hippuric acid, urea, and other substances.

[3] The ethyl acetate after separation from the watery layer of the distillate may be dried over calcium chloride and used again.

the sodium chloride. Transfer the solution from the separatory funnel to a round-bottomed flask and subject it to a steam distillation in the usual way. A *slow* current of steam should be used while the ethyl acetate is being distilled off and later a more rapid current may be employed. The distillation should be continued for twenty minutes. Now add about 0.1 gram of charcoal to the aqueous solution which is heated to boiling and filtered *hot.* Evaporate the solution in a *weighed* Jena glass dish on a water-bath until the volume of the solution is reduced to about 3 c.c. Stand the dish in a warm place until evaporation is complete and a crystalline residue remains. Wash the residue, in turn, with 2 c.c. of dry ether, and 1 c.c. of water, dry it in an air-bath at 100° C. and weigh. If it is so desired the residue may be recrystallized from a little *hot* water and the melting-point determined. Pure hippuric acid melts at 187° C. Contamination with phenaceturic acid may be detected both by the melting-point and the microscopical characteristics.

VIII. Sulphur.

1. **Total Sulphates.**—*Folin's Method.*—Place 25 c.c. of urine in a 200–250 c.c. Erlenmeyer flask, add 20 c.c. of dilute hydrochloric acid[1] (1 volume of concentrated HCl to 4 volumes of water) and gently boil the mixture for 20–30 minutes. To minimize the loss of water by evaporation the mouth of the flask should be covered with a small watch glass during the boiling process. Cool the flask for 2–3 minutes in running water, and dilute the contents to about 150 c.c. by means of *cold* water. Add 10 c.c. of a 5 per cent solution of barium chloride slowly, drop by drop, to the cold solution.[2] The contents of the flask *should not be stirred or shaken* during the addition of the barium chloride. Allow the mixture to stand at least one hour, then shake up the solution and filter it through a weighed Gooch crucible.[3]

Wash the precipitate of $BaSO_4$ with about 250 c.c. of cold water, dry it in an air-bath or over a very low flame, then ignite,[4] cool and weigh.

[1] If it is desired, 50 c.c. of urine and 4 c.c. of concentrated acid may be used instead.

[2] A dropper or capillary funnel made from an ordinary calcium chloride tube and so constructed as to deliver 10 c.c. in 2–3 minutes is recommended for use in adding the barium chloride.

[3] If a Gooch crucible is not available, the precipitate of $BaSO_4$ may be filtered off upon a washed filter paper (Schleicher & Schüll's, No. 589, blue ribbon), and after washing the precipitate with about 250 c.c. of *cold* water the paper and precipitate may be dried in an air-bath or over a low flame. The ignition may then be carried out in the usual way in the ordinary platinum or porcelain crucible. In this case correction must be made for the weight of the ash of the filter paper used.

[4] Care must be taken in the ignition of precipitates in Gooch crucibles. The flame should never be applied directly to the *perforated* bottom or to the sides of the crucible,

Calculation.—Subtract the weight of the Gooch crucible from the weight of the crucible and the $BaSO_4$ precipitate to obtain the weight of the precipitate. The weight of SO_3[1] in the volume of urine taken may be determined by means of the following proportion.

$$\underset{BaSO_4}{\text{Mol. wt.}} : \underset{\text{ppt.}}{\text{Wt. of } BaSO_4} :: \underset{SO_3}{\text{Mol. wt.}} : x \text{ (wt. of } SO_3 \text{ in grams).}$$

Representing the weight of the $BaSO_4$ precipitate by y and substituting the proper molecular weights, we have the following proportion:

$$231.7 : y \; : \; : 79.5 : x \text{ (wt. of } SO_3 \text{ in grams in the quantity of urine used).}$$

Calculate the quantity of SO_3 in the twenty-four-hour specimen of urine.

To express the result in *percentage* of SO_3 simply divide the value of x, as just determined, by the quantity of urine used.

2. **Inorganic Sulphates.**—*Folin's Method.*—Place 25 c.c. of urine and 100 c.c. of water in a 200–250 c.c. Erlenmeyer flask and acidify the diluted urine with 10 c.c. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). In case the urine is dilute 50 c.c. may be used instead of 25 c.c. and the volume of water reduced proportionately. Add 10 c.c. of 5 per cent barium chloride slowly, drop by drop, to the cold solution and from this point proceed as indicated in the method for the determination of Total Sulphates, page 378.

Calculate the quantity of inorganic sulphates, expressed as SO_3, in the twenty-four-hour urine specimen.

Calculation.—Calculate according to the directions given under Total Sulphates, above.

3. **Ethereal Sulphates.**—*Folin's Method.*—Place 125 c.c. of urine in an Erlenmeyer flask of suitable size, dilute it with 75 c.c. of water and acidify the mixture with 30 c.c. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). To the *cold* solution add 20 c.c. of a 5 per cent solution of barium chloride, drop by drop.[2] Allow the mixture to stand about one hour, then filter

since such manipulation is invariably attended by mechanical losses. The crucibles should always be provided with *lids* and *tight bottoms* during the ignition. In case porcelain Gooch crucibles, whose bottoms are not provided with a non-perforated cap, are used, the crucible may be placed upon the lid of an ordinary platinum crucible during ignition. The lid should be supported on a triangle, the crucible placed upon the lid and the flame applied to the improvised bottom. Ignition should be complete in 10 minutes if no organic matter is present.

[1] It is considered preferable by many investigators to express all sulphur values in terms of S rather than SO_3.

[2] See note (2) at the bottom of page 378.

it through a dry filter paper.[1] Collect 125 c.c. of the filtrate and boil it gently for at least one-half hour. Cool the solution, filter off the precipitate of $BaSO_4$, wash, dry and ignite it according to the directions given on page 378.

Calculation.—The weight of the $BaSO_4$ precipitate should be multiplied by 2 since only one-half (125 c.c.) of the total volume (250 c.c.) of fluid was precipitated by the barium chloride. The remaining calculation should be made according to directions given under Total Sulphates, page 378.

Calculate the quantity of ethereal sulphates, expressed as SO_3, in the twenty-four-hour urine specimen.

4. **Total Sulphur.**—*Benedict's Method.*[2]—Ten cubic centimeters of urine are measured into a *small* (7–8 c.c.) porcelain evaporating dish and 5 c.c.[3] of Benedict's sulphur reagent[4] added. The contents of the dish are evaporated over a free flame which is regulated to keep the solution just below the boiling-point, so that there can be no loss through spattering. When dryness is reached the flame is raised slightly until the entire residue has blackened. The flame is then turned up in two stages to the full heat of the bunsen burner and the contents of the dish thus *heated to redness for ten minutes after the black residue (which first fuses) has become dry.* This heating is to decompose the last traces of nitrate (and chlorate). The flame is then removed and the dish allowed to cool more or less completely. Ten to twenty cubic centimeters of dilute (1:4) hydrochloric acid is then added to the residue in the dish, which is then warmed gently until the contents have completely dissolved and a perfectly clear, sparkling solution is obtained. This dissolving of the residue requires scarcely two minutes. With the aid of a stirring rod the solution is washed into[5] a small Erlenmeyer flask, diluted with cold, distilled water to 100–150 c.c., 10 c.c. of 10 per cent barium chloride solution added drop by drop, and the solution allowed to stand for about an hour. It is then shaken up and filtered as usual through a weighed Gooch crucible.

Calculation.—Make the calculation according to directions given

[1] This precipitate consists of the inorganic sulphates. If it is desired, this $BaSO_4$ precipitate may be collected in a Gooch crucible or on an ordinary quantitative filter paper and a determination of inorganic sulphates made, using the same technique as that suggested on p. 378. In this way we are enabled to determine the inorganic and ethereal sulphates in the same sample of urine.

[2] Benedict: *Journal of Biological Chemistry*, VI, p. 363, 1909.

[3] If the urine is concentrated the quantity should be slightly increased.

[4] Crystallized copper nitrate, sulphur-free or of known sulphur content. 200 grams
Sodium or potassium chlorate ..... 50 grams
Distilled water to ..... 1000 c.c.

[5] Sometimes the porcelain glaze cracks during heating, in which case the solution should be filtered into the flask.

under Total Sulphates, p. 378. Calculate the quantity of sulphur, expressed as SO_3 or S, present in the twenty-four-hour urine specimen.

5. **Total Sulphur.**—*Osborne-Folin Method.*—Place 25 c.c. of urine[1] in a 200–250 c.c. *nickel* crucible and add about 3 grams of sodium peroxide. Evaporate the mixture to a syrup upon a steam water-bath and heat it carefully over an alcohol flame until it solidifies (15 minutes). Now remove the crucible from the flame and allow it to cool. Moisten the residue with 1–2 c.c. of water,[2] sprinkle about 7–8 grams of sodium peroxide over the contents of the crucible and fuse the mass over an alcohol flame for about 10 minutes. Allow the crucible to cool for a few minutes, add about 100 c.c. of water to the contents and heat at least one-half hour over an alcohol flame to dissolve the alkali and decompose the sodium peroxide. Next rinse the mixture into a 400–450 c.c. Erlenmeyer flask, by means of hot water, and dilute it to about 250 c.c. Heat the solution nearly to the boiling-point and add concentrated hydrochloric acid slowly until the nickelic oxide, derived from the crucible, is just brought into solution.[3] A few minutes boiling should now yield a *clear* solution. In case too little peroxide or too much water was added for the final fusion a clear solution will not be obtained. In this event cool the solution and remove the insoluble matter by filtration.

To the clear solution add 5 c.c. of very dilute alcohol (about 18–20 per cent) and continue the boiling for a few minutes. The alcohol is added to remove the chlorine which was formed when the solution was acidified. Add 10 c.c. of a 10 per cent solution of barium chloride, slowly, drop by drop,[4] to the liquid. Allow the precipitated solution to stand in the cold *two days* and then filter and continue the manipulation according to the directions given under Total Sulphates, page 378.

Calculation.—Make the calculation according to directions given under Total Sulphates, p. 378. Calculate the quantity of sulphur, expressed as SO_3 or S, present in the twenty-four-hour urine specimen.

6. **Total Sulphur.**—*Sodium Hydroxide and Potassium Nitrate Fusion Method.*—Place 25 c.c. of urine in a *silver* crucible and evaporate to a thick syrup on a water-bath. Add 10 grams of sodium hydroxide and 2 grams of potassium nitrate to the residue and fuse the mass, over an alcohol flame, until all organic matter has disap-

[1] If the urine is very dilute 50 c.c. may be used.

[2] This moistening of the residue with a small amount of water is very essential and should not be neglected.

[3] About 18 c.c. of acid is required for 8 grams of sodium peroxide.

[4] See note (2) at the bottom of page 378.

peared and the fused mixture is clear. Cool the mixture, transfer it to a casserole by means of hot water, acidify slightly with hydrochloric acid and evaporate it to dryness on a water-bath. Moisten the

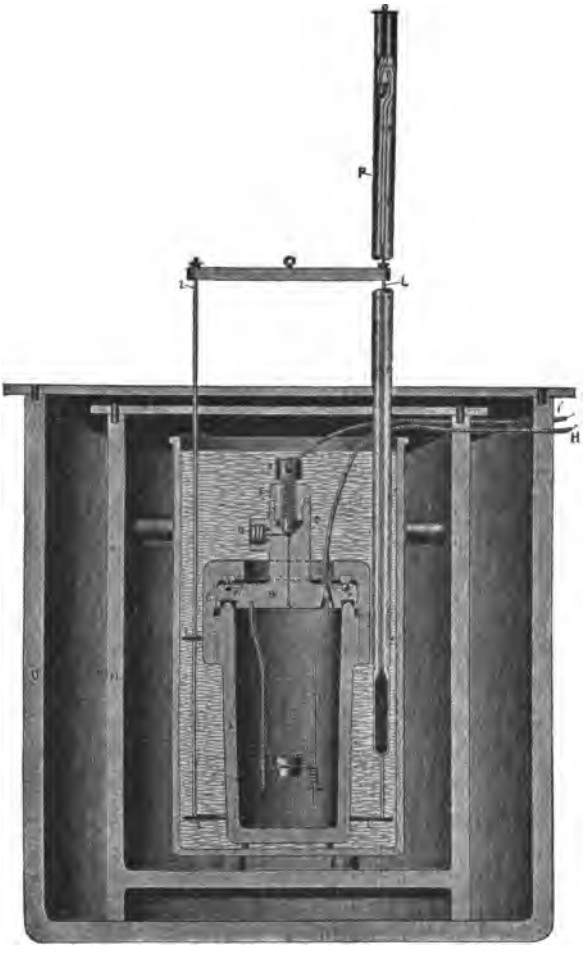


FIG. 124.—BERTHELOT-ATWATER BOMB CALORIMETER. (CROSS-SECTION OF APPARATUS AS READY FOR USE.)

A, Steel cup or bomb proper; C, collar of steel; G, opening through which oxygen is forced into the bomb; H and I′, insulated wires which serve to conduct an electric current for igniting the substance which is held in the small capsule; L, a stirrer which serves to keep the water surrounding the bomb in motion and insures the equalization of temperature; P, a delicate thermometer which shows the rise in temperature of the water surrounding the bomb.

residue with a few drops of dilute hydrochloric acid and bring it into solution with hot water. Filter, heat the filtrate to boiling, and immediately precipitate it by the addition of 10 c.c. of a 10 per cent solution

of barium chloride, adding the solution slowly, drop by drop. Allow the precipitated solution to stand 2 hours and filter while *cold*. Ignite, weigh, and calculate according to directions given under Total Sulphates, p. 378.

Compute the quantity of sulphur, expressed as SO_3 or S, present in the twenty-four-hour urine specimen.

7. **Total Sulphur.**—*Sherman's Compressed Oxygen Method.*[1]—Evaporate as much urine on an absorbent filter block[2] at 55° C. as the block will conveniently absorb and burn the block so prepared in a bomb-calorimeter[3] using 25–30 atmospheres of oxygen. Connect the bomb with a wash-bottle containing water, and allow the gas to bubble through the liquid until the high pressure within the apparatus has been reduced to atmospheric pressure. Now open the bomb and thoroughly rinse the interior, using water from the wash-bottle for the first rinsing. Dissolve any ash found in the combustion capsule in hydrochloric acid and add this solution to the main solution. Evaporate to 150 c.c., filter, and cool the filtrate. Add 10 c.c. of a 5 per cent solution of barium chloride to the *cold* filtrate, slowly, drop by drop.[4] The contents of the flask should not be stirred or shaken during the addition of the barium chloride. Allow the mixture to stand at least one hour, then shake up the solution and filter it through a weighed Gooch crucible. Manipulate the precipitate of $BaSO_4$ according to directions given under Total Sulphates, page 378.

Calculate the quantity of sulphur, expressed as SO_3 or S, present in the twenty-four-hour urine specimen.

IX. Phosphorus.

1. **Total Phosphates.**—*Uranium Acetate Method.*—To 50 c.c. of urine in a small beaker or Erlenmeyer flask add 5 c.c. of a special sodium acetate solution[5] and heat the mixture to the boiling-point. From a burette, run into the hot mixture, drop by drop, a standard solution of uranium acetate[6] until a precipitate ceases to form and a

[1] See Sherman's Organic Analysis, p. 19.

[2] Only a small amount of urine should be added at one time, it being necessary to make several evaporations before the block contains sufficient urinary residue to proceed with the combustion.

[3] The Berthelot-Atwater apparatus (Fig. 124, page 382) is well adapted to this purpose.

[4] See note (2) at the bottom of page 378.

[5] The sodium acetate solution is prepared by dissolving 100 grams of sodium acetate in 800 c.c. of distilled water, adding 100 c.c. of 30 per cent acetic acid to the solution, and making the volume of the mixture up to 1 liter with water.

[6] This uranium acetate solution may be prepared by dissolving 35.461 grams of uranium acetate in one liter of water. One c.c. of such a solution should be equivalent to 0.005 gram of P_2O_5, phosphoric anhydride. This solution may be standardized as

drop of the mixture when removed by means of a glass rod and brought in contact with a drop of a solution of potassium ferrocyanide on a porcelain test-tablet produces instantaneously a brownish-red coloration.[1] Take the burette reading and calculate the P_2O_5 content of the urine under examination.

Calculation.—Multiply the number of cubic centimeters of uranium acetate solution used by 0.005 to determine the number of *grams* of P_2O_5 in the 50 c.c. of urine used. To express the result in *percentage* of P_2O_5 multiply the value just obtained by 2, *e. g.*, if 50 c.c. of urine contained 0.074 gram of P_2O_5 it would be equivalent to 0.148 per cent.

Calculate, in terms of P_2O_5, the total phosphate content of the twenty-four-hour urine specimen.

2. **Earthy Phosphates.**—To 100 c.c. of urine in a beaker add an excess of ammonium hydroxide and allow the mixture to stand 12–24 hours. Under these conditions the phosphoric acid in combination with the alkaline earths, calcium and magnesium, is precipitated as phosphates of these metals. Collect the precipitate on a filter paper and wash it with very dilute ammonium hydroxide. Pierce the paper, and remove the precipitate by means of hot water. Bring the phosphates into solution by adding a small amount of dilute acetic acid to the warm solution. Make the volume up to 50 c.c. with water, add 5 c.c. of sodium acetate solution, and determine the P_2O_5 content of the mixture according to the directions given under the previous method.

Calculation.—Multiply the number of cubic centimeters of uranium acetate solution used by 0.005 to determine the number of *grams* of P_2O_5 in the 100 c.c. of urine used. Since 100 c.c. of urine was taken this value also expresses the *percentage* of P_2O_5 present.

Calculate the quantity of earthy phosphates, in terms of P_2O_5, present in the twenty-four-hour urine specimen.

The quantity of phosphoric acid present in combination with the *alkali* metals may be determined by subtracting the content of earthy phosphates from the total phosphates.

Total Phosphorus.—*Sodium Hydroxide and Potassium Nitrate Fusion Method.*—Place 25 c.c. of urine in a large silver crucible and

follows: To 50 c.c. of a standard solution of disodium hydrogen phosphate, of such a strength that the 50 c.c. contains 0.1 gram of P_2O_5, add 5 c.c. of the sodium acetate solution mentioned above, and titrate with the uranium solution to the correct end-reaction as indicated in the method proper. Inasmuch as 1 c.c. of the uranium solution should precipitate 0.005 gram of P_2O_5, exactly 20 c.c. of the uranium solution should be required to precipitate 50 c.c. of the standard phosphate solution. If the two solutions do not bear this relation to each other they may be brought into proper relation by diluting the uranium solution with distilled water or by increasing its strength.

[1] A 10 per cent solution of potassium ferrocyanide is satisfactory.

evaporate to a syrup on a water-bath. Add 10 grams of NaOH and 2 grams of KNO_3 to the residue and fuse the mass until all organic matter has disappeared and the fused mixture is clear. Cool the mixture, transfer it to a casserole by means of hot water, acidify the solution slightly with pure nitric acid, and evaporate to dryness on a water-bath. Moisten the residue with a few drops of dilute nitric acid, dissolve it in hot water, and transfer to a beaker. Now add an equal volume of molybdic solution[1] and keep the mixture at 40° C. for twenty-four hours. Filter off the precipitate, wash it with dilute molybdic solution, and dissolve it in dilute ammonia. Add dilute hydrochloric acid to the solution, being careful to leave the solution distinctly ammoniacal. Magnesia mixture[2] (10–15 c.c.) should now be added and after stirring thoroughly and making strongly ammoniacal with concentrated ammonia the solution should be allowed to stand in a cool place for twenty-four hours. Filter off the precipitate, wash it free from chlorine by means of dilute ammonia (1:5), dry, incinerate, and weigh, as magnesium pyrophosphate, $Mg_2P_2O_7$, in the usual manner.

In this method the phosphoric acid of the urine is precipitated as *ammonium magnesium phosphate* and in the process of incineration this body is transformed into *magnesium pyrophosphate.*

Calculation.—The quantity of phosphorus, expressed in terms of P_2O_5, in the volume of urine taken may be determined by means of the following proportion:

$$\overset{\text{Mol. wt.}}{Mg_2P_2O_7} : \overset{\text{Wt. of ppt.}}{Mg_2P_2O_7} :: \overset{\text{Mol. wt.}}{P_2O_5} : x \text{ (wt. of } P_2O_5 \text{ in grams).}$$

If y represents the weight of the $Mg_2P_2O_7$ precipitate and we make the proper substitution we have the following proportion:

$$221.1 : y :: 140.9 : x \text{ (wt. of } P_2O_5\text{, in grams, in the quantity of urine used.)}$$

To express the result in *percentage* of P_2O_5 simply divide the value of x, as just determined, by the quantity of urine used.

X. Creatinine.

Folin's Colorimetric Method.—This method is based upon the characteristic property possessed alone by creatinine, of yielding a certain definite color-reaction in the presence of picric acid in alkaline

[1] Directions for the preparation of the solution are given on p. 56.
[2] Directions for the preparation of magnesia mixture may be found on p. 289.

solution. The procedure is as follows: Place 10 c.c. of urine in a 500 c.c. volumetric flask, add 15 c.c. of a *saturated* solution of picric acid and 5 c.c. of a 10 per cent solution of sodium hydroxide, shake thoroughly and allow the mixture to stand for 5 minutes. During this interval pour a little N/2 potassium bichromate solution[1] into each of the two cylinders of the colorimeter (Duboscq's) and carefully adjust the depth of the solution in one of the cylinders to the 8 mm. mark. A few preliminary colorimetric readings may now be made with the solution in the other cylinder, in order to insure greater accuracy in the subsequent examination of the solution of unknown strength. Obviously the two solutions of potassium bichromate are identical in color and in their examination no two readings should differ more than 0.1–0.2 mm. from the true value (8 mm.). Four or more readings should be made in each case and an average taken of all of them *exclusive* of the first reading, which is apt to be less accurate than the succeeding readings. In time as one becomes proficient in the technique it is perfectly safe to take the average of the *first two readings.*

At the end of the 5-minute interval already mentioned, the contents of the 500 c.c. flask are diluted to the 500 c.c. mark, the bichromate solution is thoroughly rinsed out of one of the cylinders, and replaced with the solution thus prepared and a number of colorimetric readings are *immediately* made.

Ordinarily 10 c.c. of urine is used in the determination by this method, but if the content of creatinine is above 15 mg. or below 5 mg. the determination should be repeated with a volume of urine selected according to the content or creatinine. This variation in the volume of urine according to the content of creatinine is quite essential, since the method loses in accuracy when more than 15 mg. or less than 5 mg. of creatinine is present in the solution of unknown strength.

Calculation.—By experiment it has been determined that 10 mg. of pure creatinine, when brought into solution and diluted to 500 c.c. as explained in the above method, yields a mixture 8.1 mm. of which possesses the same colorimetric value as 8 mm. of a N/2 solution of potassium bichromate. Bearing this in mind the computation is readily made by means of the following proportion in which y represents the number of mm. of the solution of unknown strength equivalent to the 8 mm. of the potassium bichromate solution:

$y : 8.1 : : 10 : x$ (mgs. of creatinine in the quantity of urine used).

[1] This solution contains 24.55 grams of potassium bichromate to the liter.

This proportion may be used for the calculation no matter what volume of urine (5, 10, or 15 c.c.) is used in the determination. The 10 represents 10 *mg. of creatinine* which gives a color equal to 8.1 mm., whether dissolved in 5, 10, or 15 c.c. of fluid.

Calculate the quantity of creatinine in the twenty-four-hour urine specimen.

XI. Creatine.

Folin-Benedict and Myers Method.[1]—To 20 c.c. of urine in a 50 c.c. volumetric flask, add 20 c.c. of normal hydrochloric acid and place the flask in an autoclave at a temperature of 117–120° C. for one-half hour. Add distilled water until the volume of the acid-urine mixture is exactly 50 c.c., close the flask by means of a stopper, and shake it thoroughly. Approximately neutralize 25 c.c. of this mixture, introduce it into a 500 c.c. volumetric flask and determine its creatinine content according to Folin's Method (see p. 385).

Calculation.—Calculate as explained on p. 386, and from this value subtract the value for the original content of creatinine *before hydrolysis*. The difference between these two values will be the creatine content of the original urine *in terms of creatinine.*

XII. Indican.

Ellinger's Method.—This method for the quantitative determination of indican is based upon the principle underlying Jaffe's test for the *detection* of indican (see p. 275). The method is as follows: To 50 c.c. of urine[2] in a small beaker of casserole add 5 c.c. of basic lead acetate solution, mix thoroughly, and filter. Transfer 40 c.c. of the filtrate to a separatory funnel, add an equal volume of Obermayer's reagent (see p. 275) and 20 c.c. of chloroform, and extract in the usual manner. This extraction with chloroform should be repeated until the chloroform solution remains colorless. Now filter the combined chloroform extracts through a *dry* filter paper into a *dry* Erlenmeyer flask. Distil off the chloroform, heat the residue on a boiling water-bath for 5 minutes in the open flask, and wash the dried residue with hot water.[3] Add 10 c.c. of concentrated sulphuric acid to the washed

[1] Benedict and Myers: *Am. J. Phys.*, XVIII, p. 397, 1907.

[2] If the urine under examination is neutral or alkaline in reaction it should be made faintly acid with acetic acid before adding the basic lead acetate.

[3] The washing should be continued until the wash water is no longer colored. Ordinarily two or three washings are sufficient. It a separation of indigo particles takes place during the washing process, the wash water should be filtered, the indigo extracted with chloroform, and the usual method applied from this point.

residue, heat on the water-bath for 5–10 minutes, dilute with 100 c.c. of water, and titrate the blue solution with a very dilute solution of potassium permanganate.[1] The end-point is indicated by the dissipation of all the blue color from the solution and the formation of a pale yellow color.

Beautiful plates of indigo blue sometimes appear in the chloroform extract of urines containing abundant indican. In urines preserved by thymol the determination of indican is interfered with. Care should be taken, therefore, to make the indican determination upon fresh urine, before the addition of the preservative.

Calculation.—Ellinger claims that *one-sixth* of the amount determined must be *added* to the value obtained by titration in order to secure accurate data. This correction should always be made.

XIII. Chlorides.

1. **Clark's Modification of Dehn's Method.**[2]—In this method the organic compounds, that hold the chlorine too firmly for its quantitative precipitation with argentic nitrate, are destroyed by oxidation with sodium peroxide. Sodium peroxide in the presence of water gives off nascent oxygen according to the following equation:

$$Na_2O_2 + H_2O = 2NaOH + O.$$

The oxygen then attacks the organic matter and the chlorine is left as sodium chloride. The procedure is as follows: To 10 c.c. of urine in a 75–100 c.c. casserole, add 1.0–1.2 gram of sodium peroxide and evaporate the mixture to dryness on a boiling water-bath. In case the residue is not pure white, thus indicating that insufficient sodium peroxide has been added, the residue should be moistened with distilled water, additional sodium peroxide added, and the mixture again evaporated to dryness. When the oxidation is complete, treat the mass with 10–20 c.c. of distilled water and stir until it has practically all been brought into solution. Then introduce a bit of litmus paper and add dilute nitric acid (1 : 1) until the litmus paper turns red and *all effervescence ceases*. Now place the casserole on a hot plate or on a gauze and heat the contents almost to the boiling-point.[3] To

[1] A "stock solution" of potassium permanganate containing 3 grams per liter should be prepared, and when needed for titration purposes a suitable volume of this solution should be diluted with 40 volumes of water. The potassium permanganate solution should be standardized with pure indigo.

[2] Private communication to the author from Mr. S. C. Clark.

[3] If there is a slight precipitate, due to silicic acid from the casserole, this is filtered off and the filtrate collected in a 200 c.c. beaker.

the hot solution add a standard solution of argentic nitrate (see page 390) in slight excess.[1] Filter off the silver chloride while the solution is still hot and wash the precipitate thoroughly with distilled water. To the filtrate, add 1 c.c. of a saturated solution of ferric ammonium sulphate and then titrate with a standard solution of ammonium thiocyanate (see page 390) until the clear, slightly yellow fluid (or the opalescent, milky fluid, in case there is much excess of argentic nitrate) changes to a slight reddish-brown color. The color of the end-point varies with the individual. The exact end-point reached is not so important as is the securing of the *same* end-point in a series of determinations as that obtained in the standardization of the standard solutions used.

Calculation.—The standard solution of argentic nitrate should be made up so that 1 c.c. equals 0.010 gram of sodium chloride and 1 c.c. of the ammonium thiocyanate should be equivalent to 1 c.c. of the argentic nitric solution (see p. 390). Then, if the number of cubic centimeters of ammonium thiocyanate used be subtracted from the number of cubic centimeters of argentic nitrate, the difference is the number of cubic centimeters of argentic nitrate actually used in the precipitation of chlorine as silver chloride. This number, multiplied by 0.010, gives the weight in *grams* of the sodium chloride in the 10 c.c. of urine used. If it is desired to express the result in percentage of sodium chloride, move the decimal point *one* place to the right.

In a similar manner the weight or percentage of *chlorine* may be computed, using the factor 0.006 as explained in Mohr's method, below. Calculate the quantity of sodium chloride and of chlorine in the twenty-four-hour urine specimen.

2. **Mohr's Method.**—To 10 c.c. of urine in a small platinum or porcelain crucible or dish add about 2 grams of chlorine-free potassium nitrate and evaporate to dryness at 100° C. (The evaporation may be conducted over a low flame provided care is taken to prevent loss by spurting.) By means of crucible tongs hold the crucible or dish over a free flame until all carbonaceous matter has disappeared and the fused mass is slightly yellow in color. Cool the residue somewhat and bring it into solution in a small amount (15–25 c.c.) of distilled water acidified with about 10 drops of nitric acid. Transfer the solution to a small beaker, being sure to rinse out the crucible or dish very carefully. Test the reaction of the fluid, and if not already

[1] This point is most easily recognized by keeping the solution hot and in constant agitation while adding the argentic nitrate so that the silver chloride formed coagulates and sinks, leaving a clear, supernatant fluid.

acid in reaction to litmus, render it slightly acid with nitric acid. Now neutralize the solution by the addition of calcium carbonate[1] in substance, add 2–5 drops of neutral potassium chromate solution to the mixture, and titrate with a standard argentic nitrate solution.[2]

This standard solution should be run in from a burette, stirring the liquid in the beaker after each addition. The end-reaction is reached when the yellow color of the solution changes to a slight *orange-red.* At this point take the burette reading and compute the percentage of chlorine and sodium chloride in the urine examined.

Calculation.—Since 1 c.c. of the standard argentic nitrate solution is equivalent to 0.010 gram of sodium chloride, to obtain the *weight,* in grams, of the *sodium chloride* in the 10 c.c. of urine used multiply the number of cubic centimeters of standard solution used by 0.010. If it is desired to express the result in *percentage* of sodium chloride move the decimal point *one place* to the *right.*

To obtain the *weight,* in grams, of the chlorine in the 10 c.c. of urine used multiply the number of cubic centimeters of standard solution used by 0.006, and if it is desired to express the result in *percentage* of chlorine move the decimal point *one place* to the *right.*

Calculate the quantity of sodium chloride and chlorine in the twenty-four-hour urine specimen.

3. **Volhard-Arnold Method.**—Place 10 c.c. of urine in a 100 c.c. volumetric flask, add 20–30 drops of nitric acid (sp. gr. 1.2) and 2 c.c. of a cold saturated solution of ferric alum. If necessary, at this point a few drops of an 8 per cent solution of potassium permanganate may be added to dissipate the red color. Now slowly run in the standard argentic nitrate[2] solution (20 c.c. is ordinarily used) until all the chlorine has been precipitated and an *excess* of the argentic nitrate solution is present, continually shaking the mixture during the addition of the standard solution. Allow the flask to stand 10 minutes, then fill it to the 100 c.c. graduation with distilled water and *thoroughly mix* the contents. Now filter the mixture through a *dry* filter paper, collect 50 c.c. of the filtrate and titrate it with standardized ammonium thiocyanate solution.[3] The first permanent tinge of brown indicates

[1] The cessation of effervescence and the presence of some undecomposed calcium carbonate at the bottom of the vessel are the indications of neutralization.

[2] Standard argentic nitrate solution may be prepared by dissolving 29.060 grams of argentic nitrate in 1 liter of distilled water. Each cubic centimeter of this solution is equivalent to 0.010 gram of sodium chloride or to 0.006 gram of chlorine.

[3] This solution is made of such a strength that 1 c.c. of it is equal to 1 c.c. of the standard argentic nitrate solution used. To prepare the solution dissolve 12.9 grams of ammonium thiocyanate, NH_4SCN, in a little less than a liter of water. In a small flask place 20 c.c. of the standard argentic nitrate solution, 5 c.c. of the ferric alum solution and 4 c.c. of

the end-point. Take the burette reading and compute the weight of sodium chloride in the 10 c.c. of urine used.

Calculation.—The number of cubic centimeters of ammonium thiocyanate solution used indicates the excess of standard argentic nitrate solution in the 50 c.c. of filtrate titrated. Multiply this reading by 2, inasmuch as only one-half of the filtrate was employed, and subtract this product from the number of cubic centimeters of argentic nitrate (20 c.c.) originally used, in order to obtain the actual number of cubic centimeters of argentic nitrate utilized in the precipitation of the chlorides in the 10 c.c. of urine employed.

To obtain the weight in grams of the sodium chloride in the 10 c.c. of urine used, multiply the number of cubic centimeters of the standard argentic nitrate solution, actually utilized in the precipitation, by 0.010. If it is desired to express the result in *percentage* of sodium chloride move the decimal point *one* place to the *right.*

In a similar manner the weight, or percentage of *chlorine* may be computed using the factor 0.006 as explained in Mohr's method, page 389.

Calculate the quantity of sodium chloride and chlorine in the twenty-four-hour urine specimen.

XIV. Acetone and Diacetic Acid.

1. **Folin-Hart Method.**—This method serves the same purpose as the Messinger-Huppert Method, *i. e.*, the determination of both acetone and diacetic acid in terms of acetone. It is, however, much simpler and less time-consuming. The method includes the transformation of the diacetic acid into acetone and carbon dioxide by means of heat and the subsequent removal of the acetone thus formed, as well as the preformed acetone, by means of an air current as first suggested by Folin (see p. 393). The procedure is as follows: Introduce into a wide-mouthed bottle 200 c.c. of water, an accurately measured excess of N/10 iodine solution[1] and an excess of 40 per cent

nitric acid (sp. gr. 1.2), add water to make the total volume 100 c.c. and thoroughly mix the contents of the flask. Now run in the ammonium thiocyanate solution from a burette until a permanent brown tinge is produced. This is the end-reaction and indicates that the last trace of argentic nitrate has been precipitated. Take the burette reading and calculate the amount of water necessary to use in diluting the ammonium thiocyanate in order that 10 c.c. of this solution may be exactly equal to 10 c.c. of the argentic nitrate solution. Make this dilution and titrate again to be certain that the solution is of the proper strength.

[1] Proceed as follows in order to obtain a rough idea regarding the amount of N/10 iodine solution to be used: Introduce into a test-tube 10 c.c. of the urine under examination and 1 c.c. of a solution of ferric chloride made by dissolving 100 grams of ferric chloride

potassium hydroxide. Prepare an aerometer cylinder containing alkaline hypoiodite solution to absorb any acetone which may be present in the air of the laboratory, and between the cylinder and bottle suspend a test-tube about two inches in diameter. This large test-tube should contain 20 c.c. of the urine under examination, 10 drops of a 10 per cent solution of phosphoric acid, 10 grams of sodium chloride, and a little petroleum, and should be raised sufficiently high to facilitate the easy application of heat to its bottom portion. The connections on the side of the tube should be provided with bulb-tubes containing cotton. When the apparatus is arranged as described, it should be connected with a Chapman pump and an air current passed through for twenty-five minutes. During this period the contents of the test-tube are heated just to the boiling-point and after an interval of five minutes again heated in the same manner. By this means the diacetic acid is converted into acetone and at the end of the twenty-five-minute period this acetone, as well as the preformed acetone, will have been removed from the urine to the absorption bottle and there retained as iodoform.

The contents of the absorption bottle should now be acidified with concentrated hydrochloric acid,[1] and titrated with N/10 sodium thio sulphate and starch as in the Messinger-Huppert method (see below).

2. **Messinger-Huppert Method.**[2]—Place 100 c.c. of urine in a distillation flask and add 2 c.c. of 50 per cent acetic acid. Connect the flask with a condenser, properly arrange a receiver, attach a terminal series of bulbs containing water, and distil over about nine-tenths of the urine mixture. Remove the receiver, attach another, and subject the residual portion of the mixture to a second distillation. Test this fluid for acetone and if the presence of acetone

in 100 c.c. of distilled water. After permitting the mixture to stand for two minutes, compare the color with that of an equal volume of the ferric chloride solution in a test-tube of similar diameter. If the two solutions be of approximately the same color intensity, 20 c.c. of the urine under examination will yield sufficient acetone to require nearly 10 c.c. of N/10 iodine solution. In case the mixture is darker in color than is the ferric chloride solution, the former should be diluted with distilled water until it is of approximately the same intensity as the ferric chloride solution. From this data the amount of N/10 iodine solution required may be roughly estimated by means of the following table:

Urine c.c.	Ferric chloride.	Water.	N/10 Iodine required c.c.
10	1	..	10
10	1	10	20
10	1	20	35
10	1	30	50

[1] An excess of iodine is indicated by the development of a brown color.

[2] This method serves to determine *both* acetone and diacetic acid in *terms* of *acetone*.

is indicated add about 100 c.c. of water to the residue and again distil. Treat the united acetone distillates with 1 c.c. of dilute (12 per cent) sulphuric acid and redistil, collecting this second distillate in a glass-stoppered flask. During distillation, however, the glass stopper is replaced by a cork with a double perforation, the glass tube from one perforation passing to the condenser, while the bulbs containing water, before mentioned, are attached by means of the tube in the other perforation. Allow the distillation process to proceed until practically all of the fluid has passed over, then remove the receiving flask and insert the glass stopper. Now treat the distillate carefully with 10 c.c. of a N/10 solution of iodine and add sodium hydroxide solution, drop by drop, until the blue color is dissipated and the iodoform precipitates. Stopper the flask and shake it for about one minute, acidify the solution with concentrated hydrochloric acid, and note the production of a brown color if an excess of iodine is present. In case there is no such excess, the solution should be treated with N/10 iodine solution until an excess is obtained. Retitrate this excess of iodine with N/10 sodium thiosulphate solution until a light yellow color is observed. At this point a few cubic centimeters of starch paste should be added and the mixture again titrated until no blue color is visible. This is the end-reaction.

Calculation.—Subtract the number of cubic centimeters of N/10 thiosulphate solution used from the volume of N/10 iodine solution employed. Since 1 c.c. of the iodine solution is equivalent to 0.967 milligram of acetone, and since 1 c.c. of the thiosulphate solution is equivalent to 1 c.c. of the iodine solution, if we multiply the remainder from the above subtraction by 0.967 we will obtain the number of milligrams of acetone in the 100 c.c. of urine examined.

Calculate the quantity of acetone in the twenty-four-hour urine specimen.

XV. Acetone.

1. **Folin's Method.**—The same type of apparatus is used in this method as that described in Folin's method for the determination of ammonia (see p. 374). The procedure is as follows: Introduce 20–25 c.c. of the urine under examination into the aerometer cylinder and add 10 drops of 10 per cent phosphoric acid,[1] 8–10 grams of sodium chloride,[2] and a little petroleum. Introduce into an absorp-

[1] Oxalic acid (0.2–0.3 gram) may be substituted if desired.
[2] Acetone is insoluble in a saturated solution of sodium chloride.

tion flask,[1] such as is used in the ammonia determination (see p. 374), 150 c.c. of water, 10 c.c. of a 40 per cent solution of potassium hydroxide, and an excess of a N/10 iodine solution. Connect the flask with the aerometer cylinder, attach a Chapman pump, and permit an air current, slightly less rapid than that used for the determination of ammonia, to be drawn through the solution for 20–25 minutes. All of the acetone will, at this point, have been converted into iodoform in the absorption flask. Add 10 c.c. of concentrated hydrochloric acid (a volume equivalent to that of the strong alkali originally added), to the contents of the latter and titrate the excess of iodine by means of N/10 sodium thiosulphate solution and starch, as in the Messinger-Huppert method (see p. 392).

Folin has further made suggestions regarding the *simultaneous* determination of acetone and ammonia by the use of the same air current.[2] This is an important consideration for the clinician inasmuch as urines which contain acetone and diacetic acid are generally those from which the ammonia data are also desired. The procedure for the combination method is as follows: Arrange the ammonia apparatus as usual (see p. 374), and to the aerometer of the ammonia apparatus attach the acetone apparatus set up as described above. Regulate the air current with special reference to the determination of acetone and at the end of 20–25 minutes disconnect the acetone apparatus and complete the determination of the acetone as just described. The air current is not interrupted, and after having run one and one-half hours the ammonia apparatus is detached and the ammonia determination completed as described on page 374.

If data regarding diacetic acid are desired, the result obtained by Folin's method may be subtracted from the result obtained by the Messinger-Huppert method (see p. 392), inasmuch as the latter method determines both acetone and diacetic acid. Under all conditions the determination of acetone should be as expeditious as possible. This is essential, not only because of the fact that any diacetic acid present in the urine will become transformed into acetone, but *also* because of the rapid spontaneous decomposition of the alkaline hypoiodite solution used in the determination of the acetone. It has been claimed that alkaline hypoiodite solutions are almost completely converted into *iodate* solutions in *one-half hour*. Folin states, how-

[1] Folin's improved absorption tube (see Fig. 123, p. 375) should be used in this connection inasmuch as the original type embracing the use of a rubber stopper is unsatisfactory because of the solvent action of alkaline hypoiodite on rubber.

[2] These determinations may even be made on the *same sample* of urine if the sample is too small for the double determination.

ever, that the transformation is not so rapid as this, but he nevertheless emphasizes the necessity of rapidity of manipulation. At the same time it should be remembered that the air current must not be as rapid as for ammonia, inasmuch as the alkaline hypoiodite solution will not absorb all the acetone under those conditions.

XVI. Diacetic Acid.

1. **Folin-Hart Method.**—Arrange the apparatus as described under the Folin-Hart method for the determination of acetone and diacetic acid (see p. 391). Start the air current in the usual way and permit it to run 25 *minutes without the application of heat to the urine under examination.* Under these conditions the preformed acetone present in the solution is all removed (see p. 393). Immediately attach a freshly prepared absorption bottle or introduce fresh alkaline hypoiodite solution into the original bottle. Apply heat to the large test-tube as already described (see p. 392), in order to convert the diacetic acid into acetone, permit the air current to continue for the usual 25-minute period, and determine the diacetic acid value in terms of acetone by the usual titration procedure (see p. 394).

2. **Folin-Messinger-Huppert Method.**—Determine the combined acetone and diacetic acid, in terms of *acetone*, by the Messinger-Huppert method (see p. 392), and subsequently determine the acetone by Folin's method (see p. 393). Subtract the value determined by the second method from that obtained in the first method to secure data regarding the diacetic acid content of the urine, *in terms of acetone.*

XVII. β-Oxybutyric Acid.

1. **Shaffer's Method.**—Introduce 25–250 c.c. of urine[1] into a 500 c.c. volumetric flask and add an excess of basic lead acetate and 10 c.c. of concentrated ammonium hydroxide. Dilute the mixture to the 500 c.c. mark, shake the flask thoroughly and filter. Transfer 200 c.c. of the filtrate to an 800 c.c. Kjeldahl distilling flask, add 300–400 c.c. of water, 15 c.c. of concentrated sulphuric acid and a little talcum and distil the mixture until 200 to 250 c.c. of distillate

[1] The amount used depends upon the expected yield of β-oxybutyric acid. In the case of urines which give a strong ferric chloride reaction for diacetic acid, or when 5–10 grams or more of β-oxybutyric acid is expected, it is unnecessary to use more than 25–50 c.c. of urine. However, in case only a trace of β-oxybutyric acid is expected, the volume should be much larger as indicated. Under all conditions, the amount specified is sufficient for duplicate determinations. It is desirable to use such a volume of urine as contains the proper amount of β-oxybutyric acid to yield 25–50 milligrams of acetone.

has been collected (A).[1] To this distillate (A), which contains *acetone* (both preformed and that produced from diacetic acid), and *volatile fatty acids* is added 5 c.c. of 10 per cent potassium hydroxide and the distillate redistilled in order to remove the volatile fatty acids.[2] This second distillate (A_2) is then titrated with standard iodine and thiosulphate (see p. 394). The urine-sulphuric acid residue from which distillate A was obtained is again distilled, 400–600 c.c. of a 0.1–0.5 per cent potassium bichromate solution being added, by means of the dropping tube, during the process of distillation.[3] In adding the bichromate, care should be taken not to add it faster than the distillate collects except in cases where the boiling fluid assumes a pure green color, thus indicating that the bichromate is being used up more rapidly. After about 500 c.c. of distillate (B) has collected, 20 c.c. of a 3 per cent solution of hydrogen peroxide and a few cubic centimeters of potassium hydroxide solution are added and the mixture (B) subjected to redistillation. Distil off about 300 c.c. and titrate this distillate (B_2) as usual with iodine and thiosulphate (see p. 394).

Calculation.—The author advises the use of solutions of thiosulphate and iodine, which are a trifle stronger than N/10; *i.e.*, 103.4 N/10. Each cubic centimeter of an iodine solution of this strength is equivalent to one milligram of acetone or to 1.794 milligrams of β-oxybutyric acid. The thiosulphate solution is accepted as the standard and should be restandardized, from time to time, by a N/10 solution of potassium bi-iodate.

2. **Black's Method.**—Render 50 c.c. of the urine under examination, faintly alkaline with sodium carbonate and evaporate to one-third the original volume. Concentrate to about 10 c.c. on a water-bath, cool the residue, acidify it with a few drops of concentrated hydrochloric acid[4] and add plaster of Paris to form a thick paste. Permit the mixture to stand until it begins to "set," then break it up with a stout glass rod having a blunt end and reduce the material to the consistency of a fairly dry coarse meal.[5] Transfer the meal to a Soxhlet apparatus and extract with ether for two hours. At the end of this period evaporate the ether-extract either spontaneously or in

[1] This distilling flask should be provided with a dropping tube, by means of which water may be introduced in order to prevent the contents of the flask from becoming less than 400 c.c. in volume. Care should be taken to use a good condenser in the distillation, but it is not necessary to cool the distillate with ice.

[2] Formic acid is one of the most troublesome.

[3] Generally the addition of 0.5 gram of potassium bichromate is sufficient. In case the urine contains a high concentration of sugar or when a large volume of urine is used, it may be necessary to use 2–3 grams of the bichromate.

[4] The residue should give a distinct red color with litmus paper.

[5] Before this is accomplished it may, in some cases, be necessary to add a little more plaster of Paris.

an air current. Dissolve the residue in water, add a little bone-black, if necessary, filter until a clear solution is obtained and make up the filtrate to a known volume (25 c.c. or less) with water. The β-oxybutyric acid should then be determined by means of the polariscope.

3. **Darmstadter's Method.**—This method is based on the fact that crotonic acid is formed from β-oxybutyric acid under the influence of concentrated mineral acids. The method is as follows: Render 100 c.c. of urine slightly alkaline with sodium carbonate and evaporate nearly to dryness on a water-bath. Dissolve the residue in 150–200 c.c. of 50–55 per cent sulphuric acid, transfer the acid solution to a 1-liter distillation flask and connect it with a condenser. Through the cork of the flask introduce the stem of a dropping funnel containing water. Heat the flask gently until foaming ceases, then use a full flame and distil over about 300–350 c.c. of fluid, keeping the volume of liquid in the distillation flask constant by the addition of water from the dropping funnel as the distillate collects. Ordinarily it will take about 2–2 1/2 hours to collect this amount of distillate. Extract the distillate three times[1] with ether in a separatory funnel, evaporate the ether and heat the residue at 160° C. for a few minutes to remove volatile fatty acids. Dissolve the residue in 50 c.c. of water, filter and titrate this aqueous solution of crotonic acid with N/10 sodium hydroxide solution, using phenolphthalein as indicator.

Calculation.—One c.c. of N/10 sodium hydroxide solution equals 0.0086 gram of crotonic acid, 1 part of crotonic acid equals 1.21 part of β-oxybutyric acid, and 1 c.c. of N/10 sodium hydroxide solutions equals 0.01041 gram of β-oxybutyric acid. To compute the quantity of β-oxybutyric acid, in grams, multiply the number of cubic centimeters of N/10 sodium hydroxide solution used by 0.01041.

4. **Bergell's Method.**—Render 100–300 c.c. of sugar-free[2] urine slightly alkaline with sodium carbonate, evaporate the alkaline urine to a syrup on a water-bath, cool the syrup, rub it up with syrupy phosphoric acid (being careful to keep the mixture cool), 20–30 grams of finely pulverized, anhydrous cupric sulphate, and 20–25 grams of fine sand. Mix the mass thoroughly, place it in a paper extraction thimble[3] and extract the dry mixture with ether in a Soxhlet apparatus (Fig. 126, page 405). Evaporate the ether, dissolve the residue in about 25 c.c. of water, decolorize the fluid with animal charcoal, if

[1] Shaffer has recently called attention to the fact that it is extremely difficult to extract all of the crotonic acid if but *three* extractions are made.

[2] If sugar is present it must be removed by fermentation.

[3] The Schleicher and Schüll fat-free extraction thimble is very satisfactory.

necessary, and determine the content of β-oxybutyric acid by a polarization test.

5. **Boekelman and Bouma's Method.**—Place 25 c.c. of urine in a flask, add 25 c.c. of 12 per cent sodium hydroxide and 25 c.c. of benzoyl chloride, stopper the flask and shake it vigorously for three minutes *under cold water.* Remove the clear fluid by means of a pipette, filter it and subject it to a polarization test. Through the action of the benzoyl chloride all the lævo-rotatory substances except β-oxybutyric acid will have been removed and the lævo-rotation now exhibited by the urine will be due entirely to that acid.

XVIII. Acidity.

Folin's Method.—The *total acidity* of urine may be determined as follows: Place 25 c.c. of urine in a 200 c.c. Erlenmeyer flask and add 15–20 grams of finely pulverized potassium oxalate and 1–2 drops of a 1 per cent phenolphthalein solution to the fluid. Shake the mixture vigorously for 1–2 minutes and titrate it immediately with N/10 sodium hydroxide until a faint but unmistakable pink remains permanent on further shaking. Take the burette reading and calculate the acidity of the urine under examination.

Calculation.—If y represents the number of cubic centimeters of N/10 sodium hydroxide used and y' represents the volume of urine excreted in twenty-four hours, the *total acidity* of the twenty-four-hour urine specimen may be calculated by means of the following proportion:

$25 : y :: y' : x$ (acidity of 24-hour urine expressed in cubic centimeters of N/10 sodium hydroxide).

Each cubic centimeter of N/10 sodium hydroxide contains 0.004 gram of sodium hydroxide, and this is equivalent to 0.0063 gram of oxalic acid. Therefore, in order to express the total acidity of the twenty-four-hour urine specimen in equivalent grams of sodium hydroxide, multiply the value of x, as just determined, by 0.004, or multiply the value of x by 0.0063 if it is desired to express the total acidity in grams of oxalic acid.

XIX. Purine Bases.

1. **Welker's Modification of the Methods of Arnstein and of Salkowski.**[1]—Four hundred cubic centimeters of urine, free from protein, are treated with 100 c.c. of magnesia mixture and 600 c.c.

[1] Private communication from Dr. W. H. Welker.

of water. This is then filtered and of the clear filtrate a measured quantity (600–800 c.c.) is treated with an excess (10 c.c.) of a 3 per cent silver nitrate solution. Concentrated ammonium hydroxide is added in small quantities, with stirring, until all the chlorides have dissolved. Allow the flocculent precipitate of the silver purine compounds to settle to the bottom, then pass the supernatant liquid through the filter before disturbing the precipitate. Finally transfer the precipitate quantitatively to the paper which must be of known nitrogen content. The precipitate is washed with dilute (1 per cent) ammonium hydroxide. The paper with the precipitate is then transferred to a Kjeldahl flask and about 100 c.c. of water and a small quantity (about 0.1 gram) of magnesium oxide are added. The water is then boiled until all the ammonia has been driven off. Test the steam with litmus paper.

The material in the flask is then digested by means of the usual Kjeldahl method (see p. 375). The digestion must be watched carefully at the time the sulphuric acid reaches sufficient concentration to affect the filter paper, inasmuch as the SO_2 produced causes considerable frothing. The *total* nitrogen (purine base, uric acid and filter-paper nitrogen) is now determined in the usual way (see Kjeldahl Method, p. 375). This result *minus* the uric acid and filter-paper nitrogen will give the figure for the purine-base nitrogen.

2. **Krüger and Schmidt's Method.**—This method serves for the determination of both uric acid and the purine bases. The principle involved is the precipitation of both the uric acid and the purine bases in combination with copper oxide and the subsequent decomposition of this precipitate by means of sodium sulphide. The uric acid is then precipitated by means of hydrochloric acid and the purine bases are separated from the filtrate in the form of their copper or silver compounds. The nitrogen content of the precipitates of uric acid and purine bases is then determined by means of the Kjeldahl method (see p. 375) and the corresponding values for uric acid and purine bases calculated. The method is as follows: To 400 c.c. of albumin-free urine[1] in a liter flask,[2] add 24 grams of sodium acetate, 40 c.c. of a solution of sodium bisulphite[3] and heat the mixture to boiling. Add 40–80 c.c.[4] of a 10 per cent solution of cupric sulphate and maintain the temperature of the mixture at the boiling-point for at least

[1] If albumin is present, the urine should be heated to boiling, acidified with acetic acid, and filtered.

[2] The total volume of urine for the twenty-four hours should be sufficiently diluted with water to make the total volume of the solution 1600–2000 c.c.

[3] A solution containing 50 grams of Kahlbaum's commercial sodium bisulphite in 100 c.c. of water.

[4] The exact amount depending upon the content of the purine bases.

three minutes. Filter off the flocculent precipitate, wash it with hot water until the wash water is colorless, and return the washed precipitate to the flask by puncturing the tip of the filter paper and washing the precipitate through by means of hot water. Add water until the volume in the flask is approximately 200 c.c., heat the mixture to boiling and decompose the precipitate of copper oxide by the addition of 30 c.c. of sodium sulphide solution.[1] After decomposition is complete, the mixture should be acidified with acetic acid and heated to boiling until the separating sulphur collects in a mass. Filter the hot fluid by means of a filter-pump, wash with *hot* water, add 10 c.c. of 10 per cent hydrochloric acid and evaporate the filtrate in a porcelain dish until the total volume has been reduced to about 10 c.c. Permit this residue to stand about two hours to allow for the separation of the uric acid, leaving the purine bases in solution. Filter off the precipitate of uric acid, using a small filter paper, and wash the uric acid, with water made acid with sulphuric acid, until the total volume of the original filtrate and the wash water aggregates 75 c.c. Determine the nitrogen content of the precipitate by means of the Kjeldahl method (see p. 375), and calculate the uric acid equivalent.[2]

Render the filtrate from the uric acid crystals alkaline with sodium hydroxide, add acetic acid until faintly acid and heat to 70° C. Now add 1 c.c. of a 10 per cent solution of acetic acid and 10 c.c. of a suspension of manganese dioxide[3] to oxidize the traces of uric acid which remain in the solution. Agitate the mixture for one minute, add 10 c.c. of the sodium bisulphite solution[4] and 5 c.c. of a 10 per cent solution of cupric sulphate and heat the mixture to boiling for three minutes. Filter off the precipitate, wash it with *hot* water, and determine its nitrogen content by means of the Kjeldahl method (see p. 375). Inasmuch as the composition and proportion of the purine bases present in urine is variable, no factor can be applied. The result as regards these bases must therefore be expressed in terms of nitrogen.

[1] This is made by saturating a 1 per cent solution of sodium hydroxide with hydrogen sulphide gas and adding an equal volume of 1 per cent sodium hydroxide.

Ordinarily the addition of 30 c.c. of this solution is sufficient, but the presence of an excess of sulphide should be *proven* by adding a drop of lead acetate to a drop of the solution. Under these conditions a dark brown color will show the presence of an excess of sodium sulphide.

[2] This may be done by multiplying the nitrogen value by three and adding three and one-half milligrams to the product as a correction for the uric acid remaining in solution in the 75 c.c.

[3] Made by heating a 0.5 per cent solution of potassium permanganate with a little alcohol until it is decolorized.

[4] To dissolve the excess of manganese dioxide.

Benedict and Saiki[1] report cases in which the *total* purine nitrogen by this method was less than the uric-acid nitrogen as determined by the Folin-Shaffer method. The inaccuracy was found to lie in the Krüger and Schmidt method. To obviate this they advise the addition of 20 c.c. of glacial acetic acid for each 300 c.c. of urine employed, the acid being added before the first precipitation.

3. **Salkowski's Method.**—Place 400–600 c.c. of protein-free urine in a beaker. Introduce into another beaker 30–50 c.c. of an ammoniacal silver solution[2] with 30–50 c.c. of magnesia mixture,[3] add some ammonium hydroxide and if necessary some ammonium chloride to clear the solution. Now add this solution to the urine, stirring continually with a glass rod, and allow the mixture to stand for one-half hour. Collect the precipitate on a filter paper, wash it with dilute ammonium hydroxide, and finally wash it back into the original beaker. Suspend the precipitate in 600–800 c.c. of water, add a few drops of hydrochloric acid and decompose it by means of hydrogen sulphide. Now heat the solution to boiling, filter while hot and evaporate the filtrate to dryness on a water-bath. Extract the residue with 20–30 c.c. of hot 3 per cent sulphuric acid and allow the extract to stand twenty-four hours. Filter off the uric acid, wash it, make the filtrate ammoniacal and precipitate the purine bases again with silver nitrate. Collect this precipitate on a small-sized chlorine-free filter paper, wash, dry, and incinerate it in the usual manner. Now dissolve the ash in nitric acid and titrate with ammonium thiocyanate according to the Volhard-Arnold method (see p. 390). Calculate the content of purine bases in the urine examined, bearing in mind that in an equal mixture of the silver salts of the purine bases, such as we have here, one part of silver corresponds to 0.277 gram of nitrogen or to 0.7381 gram of the bases.

XX. Allantoin.

Paduschka-Underhill-Kleiner Method.—To 50–100 c.c. of urine in a beaker add basic lead acetate until no more precipitate forms. Filter and pass hydrogen sulphide gas through an aliquot portion of the filtrate to remove the excess of lead.[4] Filter again, drive off the hydrogen sulphide by heat and treat an aliquot portion of

[1] Benedict and Saiki: *Jour. Biol. Chem.*, VII, p. 27, 1909.

[2] Prepared by dissolving 26 grams of silver nitrate in about 500 c.c. of water, adding enough ammonium hydroxide to redissolve the precipitate which forms upon the first addition of the ammonia and making the balance of the mixture up to 1 liter with water.

[3] Directions for preparation may be found on page 289.

[4] In the original method of Paduschka sodium sulphate is used for this purpose.

the filtrate with a 10 per cent solution of silver nitrate until precipitation is complete.[1] Filter off this precipitate, wash it with water and determine its nitrogen content by means of the Kjeldahl method (see p. 375). This is the "purine nitrogen." Render an aliquot portion of the filtrate faintly alkaline,[2] with a 1 per cent solution of ammonium hydroxide and add 50–100 c.c. of a 10 per cent solution of silver nitrate. If allantoïn be present a white, flocculent precipitate will form and gradually sink to the bottom of the solution. Filter, wash the precipitate free from ammonium hydroxide by means of a 1 per cent solution of sodium sulphate and determine its nitrogen content by the Kjeldahl method (see p. 375).

XXI. Oxalic Acid.

Salkowski-Autenrieth and Barth Method.—Place the twenty-four-hour urine specimen in a precipitating jar, add an excess of calcium chloride, render the urine strongly ammoniacal, stir it well, and allow it to stand 18–20 hours. Filter off the precipitate, wash it with a small amount of water and dissolve it in about 30 c.c. of a *hot* 15 per cent solution of hydrochloric acid. By means of a separatory funnel extract the solution with 150 c.c. of ether which contains 3 per cent of alcohol, repeating the extraction four or five times with fresh portions of ether. Unite the ethereal extracts, allow them to stand for an hour in a flask, and then filter through a *dry* filter paper. Add 5 c.c. of water to the filtrate, to prevent the formation of diethyl oxalate when the solution is heated, and distil off the ether. If necessary, decolorize the liquid with animal charcoal and filter. Concentrate the filtrate to 3–5 c.c., add a little calcium chloride solution, make it ammoniacal, and after a few minutes render it slightly acid with acetic acid. Allow the acidified solution to stand several hours, collect the precipitate of calcium oxalate on a washed filter paper,[3] wash, incinerate strongly (to CaO), and weigh in the usual manner.

Calculation.—Since 56 parts of CaO are equivalent to 90 parts of oxalic acid, the quantity of oxalic acid in the volume of urine taken may be determined by multiplying the weight of CaO by the factor 1.6071.

XXII. Total Solids.

1. **Drying Method.**—Place 5 c.c. of urine in a weighed shallow dish, acidify *very slightly* with acetic acid (1–3 drops), and dry it *in*

[1] Ordinarily from 20–30 c.c. is required.
[2] Using litmus as the indicator.
[3] Schleicher and Schüll, No. 589, is satisfactory.

vacuo in the presence of sulphuric acid to constant weight. Calculate the *percentage* of solids in the urine sample and the *total solids* for the twenty-four-hour period.

Practically all the methods the technique of which includes evaporation at an increased temperature, either under atmospheric conditions or *in vacuo*, are attended with error.

2. **Calculation by Long's Coefficient.**—The quantity of solid material contained in the urine excreted for any twenty-four-hour period may be approximately computed by multiplying the second and third decimal figures of the specific gravity by 2.6. This gives us the *number of grams of solid matter in* 1 *liter of urine.* From this value the total solids for the twenty-four-hour period may easily be determined.

Calculation.—If the volume of urine for the twenty-four hours was 1120 c.c. and the specific gravity 1.018, the calculation would be as follows:

(*a*) $18 \times 2.6 = 46.8$ grams of solid matter in 1 liter of urine.

(*b*) $\frac{46.8 \times 1120}{1000} = 52.4$ grams of solid matter in 1120 c.c. of urine.

Long's coefficient was determined for urine whose specific gravity was taken at 25° C. and is probably more accurate, for conditions obtaining in America, than the older coefficient of Haeser, 2.33.

CHAPTER XXIII.

QUANTITATIVE ANALYSIS OF MILK, GASTRIC JUICE, AND BLOOD.

(a) Quantitative Analysis of Milk.

1. **Specific Gravity.**—This may be determined conveniently by means of a Soxhlet, Veith, or Quevenne *lactometer*. A lactometer reading of 32° denotes a specific gravity of 1.032. The determination should be made at about 60° F. and the lactometer reading corrected by adding or subtracting 0.1° for every degree F. above or below that temperature.

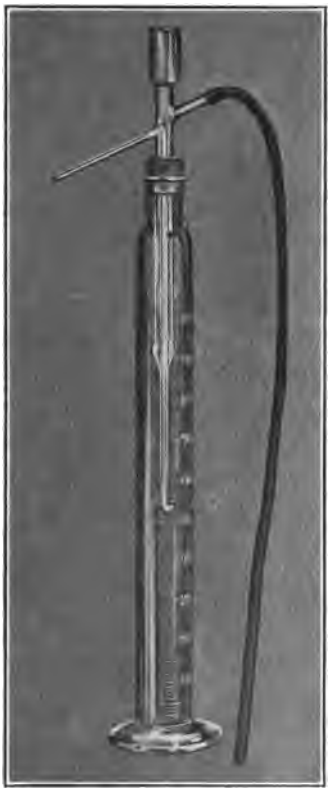


FIG. 125.—CROLL'S FAT APPARATUS.

2. **Fat.**—(*a*) *Quantitative Determination of Fat in Milk by the Meigs*[1] *Method with Modification and Improved Apparatus by Croll.*[2]—The method as stated by Dr. Meigs is: Approximately 10 c.c. of milk is carefully weighed and transferred to an ordinary 100 c.c. glass-stoppered graduated cylinder. Twenty c.c. each of distilled water and ether (0.720) are added, the ground-glass stopper tightly inserted in the bottle, and the whole shaken vigorously for five minutes. Then the bottle is carefully unstoppered, 20 c.c. 95 per cent alcohol added, the stopper reinserted and again shaken for five minutes. The bottle is now placed on a table and the contents will separate into two distinct strata, the upper of which contains practically all the fat. This stratum is carefully removed by a small pipette and transferred to a carefully weighed glass evaporating dish. The thin ether layer remaining is washed by the addition of 5 c.c. of ether. This is removed by pipetting off. This washing is repeated

[1] Original paper by Dr. Arthur V. Meigs in *Philadelphia Medical Times*, July 1, 1882.

[2] Private Communication.

four times. On each addition the sides of the bottle should carefully be washed down by the fresh ether. Finally, the pipette is rinsed with a little ether. The evaporating dish with contents is now placed on a safety water-bath and the ether evaporated. The drying is continued in a hot-air oven at a temperature below 100° C. and finally completed in a desiccator to constant weight.

Croll's modification consists of subsequent repeated extraction of the end-product of evaporation with absolute ether. The combined extracts are filtered and the small filter paper is washed repeatedly with absolute ether. The combined extracts and washings are evaporated and dried as before and then weighed.

The piece of apparatus shown in Fig. 125, p. 404 was also devised by Croll to do away with the use of the pipette. On closing the top with a finger and blowing into the mouth-piece, the upper stratum is forced out into the dish. The bottle is washed by simply pouring the ether into the tube. This lessens the possibility of accidental loss.

FIG. 126.—SOXHLET APPARATUS.

The accuracy of the method compared with that of the Soxhlet method, using the paper-coil modification and extracting until fresh portions of absolute ether gave no further trace of extractive material, is shown by the average difference on twelve samples of human milk being only 0.017 per cent less than by the Soxhlet and on seven samples cow's milk being only 0.019 per cent less. The extreme differences in case of the human milk were −0.004 per cent and −0.044 per cent and in case of the cow's milk −0.006 per cent and −0.068 per cent.

(*b*) *Adams' Paper-coil Method.*—Introduce about 5 c.c. of milk into a small beaker, quickly ascertain the weight to centigrams, stand a fat-free coil[1] in the beaker, and incline the vessel and rotate the coil

[1] Very satisfactory coils are manufactured by Schleicher and Schüll.

in order to hasten the absorption of the milk. Immediately upon the complete absorption of the milk remove the coil and again quickly ascertain the weight of the beaker. The difference in the weights of the beaker at the two weighings represents the quantity of milk absorbed by the coil. Dry the coil carefully at a temperature below 100° C. and extract it with ether for 3–5 hours in a Soxhlet apparatus (Fig. 126, p. 405). Using a safety water-bath, heat the flask containing the fat to constant weight at a temperature below 100° C.

Calculation.—Divide the weight of fat, in grams, by the weight of milk, in grams. The quotient is the *percentage of fat* contained in the milk examined.

(*c*) *Approximate Determination by Feser's Lactoscope.*—Milk is opaque mainly because of the suspended fat globules and therefore by means of the estimation of this opacity we may obtain data as to the *approximate* content of fat. Feser's lactoscope (Fig. 127) may be used for this purpose. Proceed as follows: By means of the graduated pipette accompanying the instrument introduce 4 c.c. of milk into the lactoscope. Add water gradually, shaking after each addition, and note the point at which the black lines upon the inner white glass cylinder are *distinctly* visible. Observe the point on the graduated scale of the lactoscope which is level with the surface of the diluted milk. This reading represents the *percentage of fat present in the undiluted milk.* Pure milk should contain at least 3 per cent of fat.

FIG. 127.—FESER'S LACTOSCOPE.

3. **Total Solids.**[1]—Introduce 2–5 grams of milk into a *weighed* flat-bottomed platinum dish[2] and quickly ascertain the weight to milligrams. Expel the major portion of the water by heating the *open* dish on a water-bath and continue the heating in an air-bath or water oven at 97°–100° C. until the weight is constant. (If platinum dishes are employed this residue may be used in the determination of *ash* according to the method described on p. 407.)

Calculation.—Divide the weight of the residue, in grams, by the

[1] The percentage of total solids may be calculated from the *specific gravity* and *percentage of fat* by means of the following formula which has been proposed by Richmond:

$$S = 0.25\,L + 1.2\,F + 0.14$$

S = total solids.
L = lactometer reading.
F = fat content.

[2] Lead foil dishes, costing only about one dollar per gross, make a very satisfactory substitute for the platinum dishes.

weight of milk used, in grams. The quotient is the *percentage of solids* contained in the milk examined.

4. **Ash.**—Heat the dry solids from 2–5 grams of milk, obtained according to the method just given, over a very low flame[1] until a white or light gray ash is obtained. Cool the dish in a desiccator and weigh. (This ash may be used in testing for preservatives according to directions on page 221.

5. **Proteins.**—Introduce a known weight of milk (5–10 grams) into a 500 c.c. Kjeldahl digestion flask and add 20 c.c. of concentrated sulphuric acid and about 0.2 gram of cupric sulphate. Expel the major portion of the water by heating over a low flame and finally use a full flame and allow the mixture to boil 1–2 hours. Complete the determination according to the directions given under Kjeldahl Method, page 375.

Calculation.—Multiply the *total nitrogen* content by the factor 6.37[2] to obtain the protein content of the milk examined.

6. **Caseinogen.**—Mix about 20 grams of milk with 40 c.c. of a saturated solution of magnesium sulphate and add the salt in substance until no more will dissolve. The precipitate consists of caseinogen admixed with a little fat and lacto-globulin. Filter off the precipitate, wash it thoroughly with a saturated solution of magnesium sulphate,[3] transfer the filter paper and precipitate to a Kjeldahl digestion flask, and determine the nitrogen content according to the directions given in the previous experiment.

Calculation.—Multiply the *total nitrogen* by the factor 6.37 to obtain the casein content.

7. **Lactalbumin.**—To the filtrate and washings from the determination of caseinogen, as just explained, add Almén's reagent[4] until no more precipitate forms. Filter off the precipitate and determine the nitrogen content according to the directions given under Proteins, above.

Calculation.—Multiply the *total nitrogen* by the factor 6.37 to obtain the lactalbumin content.

8. **Lactose.**—To about 350 c.c. of water in a beaker add 20 grams

[1] Great care should be used in this ignition, the dish at no time being heated above a faint redness, as chlorides may volatilize.

[2] The usual factor employed for the calculation of protein from the nitrogen content is 6.25 and is based on the assumption that proteins contain *on the average* 16 per cent of nitrogen. This special factor of 6.37 is used here to calculate the protein content from the total nitrogen, since the principal protein constituents of milk, *i. e.*, *caseinogen* and *lactalbumin*, contain 15.7 per cent of nitrogen.

[3] Preserve the filtrate and washings for the determination of lactalbumin.

[4] Almén's reagent may be prepared by dissolving 5 grams of tannin in 240 c.c. of 50 per cent alcohol and adding 10 c.c. of 25 per cent acetic acid.

of milk, mix thoroughly, acidify the fluid with about 2 c.c. of 10 per cent acetic acid and stir the acidified mixture continuously until a flocculent precipitate forms. At this point the reaction should be distinctly acid to litmus. Heat the solution to boiling for one-half hour, filter, rinse the beaker thoroughly, and wash the precipitated proteins and the adherent fat with *hot* water. Combine the filtrate and wash water and concentrate the mixture to about 150 c.c. Cool the solution and dilute it to 200 c.c. in a volumetric flask. Titrate this sugar solution according to directions given under Fehling's Method, page 362.

Calculation.—Make the calculation according to directions given under Fehling's Method, p. 362, bearing in mind that 10 c.c. of Fehling's solution is completely reduced by 0.0676 *gram of lactose.*

(b) Quantitative Analysis of Gastric Juice.

Töpfer's Method.

This method is much less elaborate than many others but is sufficiently accurate for ordinary clinical purposes. The method embraces the volumetric determination of (1) *total acidity*, (2) *combined acidity*, and (3) *free acidity*, and the subsequent calculation of (4) *acidity due to organic acids and acid salts*, from the data thus obtained.

Strain the gastric contents and introduce 10 c.c. of the strained material into each of three small beakers or porcelain dishes.[1] Label the vessels *A*, *B* and *C*, respectively, and proceed with the analysis according to the directions given below.

1. **Total Acidity.**[2]—Add 3 drops of a 1 per cent alcoholic solution of phenolphthalein[3] to the contents of vessel *A* and titrate with N/10 sodium hydroxide solution until a *dark pink* color is produced which cannot be deepened by further addition of a drop of N/10 sodium hydroxide. Take the burette reading and calculate the total acidity.

Calculation.—The total acidity may be expressed in the following ways:

1. The number of cubic centimeters of N/10 sodium hydroxide solution necessary to neutralize 100 c.c. of gastric juice.

2. The weight (in grams) of sodium hydroxide necessary to neutralize 100 c.c. of gastric juice.

[1] If sufficient gastric juice is not available it may be diluted with water or a smaller amount, *e. g.*, 5 c.c. taken for each determination.

[2] This includes free and combined acid and acid salts.

[3] One gram of phenolphthalein dissolved in 100 c.c. of 95 per cent alcohol.

3. The weight (in grams) of hydrochloric acid which the total acidity of 100 c.c. of gastric juice represents, *i. e.*, percentage of hydrochloric acid.

The forms of expression most frequently employed are 1 and 3, preference being given to the former.

In making the calculation note the number of cubic centimeters of N/10 sodium hydroxide required to neutralize 10 c.c. of the gastric juice and multiply it by 10 to obtain the number of cubic centimeters necessary to neutralize 100 c.c. of the fluid. If it is desired to express the acidity of 100 c.c. of gastric juice in terms of hydrochloric acid, by weight, multiply the value just obtained by 0.00365.[1]

2. **Combined Acidity.**[2]—Add 3 drops of sodium alizarin sulphonate solution[3] to the contents of vessel *B* and titrate with N/10 sodium hydroxide solution until a *violet* color is produced. In this titration the red color, which appears after the tinge of yellow due to the addition of the indicator has disappeared, must be entirely replaced by a *distinct violet color*. Take the burette reading and calculate the combined acidity.

Calculation.—Since the indicator used reacts to all acidities except combined acidity, in order to determine the number of cubic centimeters of N/10 sodium hydroxide necessary to neutralize the combined acidity of 10 c.c. of the gastric juice, we must subtract the burette reading just obtained from the burette reading obtained in the determination of the total acidity. The data for 100 c.c. of gastric juice may be calculated according to the directions given under Total Acidity, page 408.

3. **Free Acidity.**[4]—Add 4 drops of di-methyl-amino-azobenzene (Töpfer's reagent) solution[5] to the contents of the vessel *C* and titrate with N/10 sodium hydroxide solution until the initial red color is replaced by lemon *yellow*.[6] Take the burette reading and calculate the free acidity.

Calculation.—The indicator used reacts only to free acid, hence the number of cubic centimeters of N/10 sodium hydroxide used indicates the volume necessary to neutralize the *free acidity* of 10 c.c. of gastric juice. To determine the data for 100 c.c. of gastric juice proceed according to the directions given under Total Acidity, page 408.

[1] One c.c. of N/10 hydrochloric acid contains 0.00365 gram of hydrochloric acid.
[2] Hydrochloric acid combined with protein material.
[3] One gram of sodium alizarin sulphonate dissolved in 100 c.c. of water.
[4] Hydrochloric acid *not* combined with protein material.
[5] One-half gram dissolved in 100 c.c. of 95 per cent alcohol.
[6] If the lemon yellow color appears as soon as the indicator is added it denotes the *absence of free* acid.

4. **Acidity Due to Organic Acids and Acid Salts.**—This value may be conveniently calculated by subtracting the number of cubic centimeters of N/10 sodium hydroxide used in neutralizing the contents of vessel *C* from the number of cubic centimeters of N/10 sodium hydroxide solution used in neutralizing the contents of vessel *B*. The remainder indicates the number of cubic centimeters of N/10 sodium hydroxide solution necessary to neutralize the acidity due to organic acids and acid salts present in 10 c.c. of gastric juice. The data for 100 c.c. of gastric juice may be calculated according to directions given under Total Acidity, page 408.

(c) Quantitative Analysis of Blood.

For the methods involved in the quantitative examination of blood see Chapter XII.

APPENDIX.

Almen's Reagent.[1]—Dissolve 5 grams of tannin in 240 c.c. of 50 per cent alcohol and add 10 c.c. of 25 per cent acetic acid.

Ammoniacal Silver Solution.[2]—Dissolve 26 grams of silver nitrate in about 500 c.c. of water, add enough ammonium hydroxide to redissolve the precipitate which forms upon the first addition of the ammonium hydroxide and make the volume of the mixture up to 1 liter with water.

Arnold-Lipliawsky Reagent.[3]—This reagent consists of two definite solutions which are ordinarily preserved separately and mixed just before using. The two solutions are prepared as follows:

(*a*) One per cent aqueous solution of potassium nitrite.

(*b*) One gram of *p*-amino-acetophenon dissolved in 100 c.c. of distilled water and enough hydrochloric acid (about 2. c.c) added drop by drop, to cause the solution, which is at first yellow, to become entirely colorless. An excess of acid must be avoided.

Barfoed's Solution.[4]—Dissolve 4.5 grams of neutral, crystallized cupric acetate in 100 c.c. of water and add 1.2 c.c. of 50 per cent acetic acid.

Baryta Mixture.[5]—A mixture consisting of one volume of a saturated solution of barium nitrate and two volumes of a saturated solution of barium hydroxide.

Benedict's Solutions.[6]—*First Modification.*—Benedict's modified Fehling solution consists of two definite solutions—a cupric sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

Cupric sulphate solution = 34.65 grams of cupric sulphate dissolved in water and made up to 500 c.c.

Alkaline tartrate solution = 100 grams of anhydrous sodium carbonate and 173 grams of Rochelle salt dissolved in water and made up to 100 c.c.

[1] Ott's precipitation test, p. 315. Determination of lactalbumin, p. 407.
[2] Salkowski's method, page 401.
[3] Arnold-Lipliawsky reaction, page 326.
[4] Barfoed's test, pages 30 and 307.
[5] Isolation of urea from urine, page 263.
[6] Benedict's modifications of Fehling's test, pages 27 and 303, and Benedict's Method, page 363.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

Second Modification.—Very recently Benedict has further modified his solution and has succeeded in obtaining one which does not deteriorate upon long standing. It has the following composition:

Cupric sulphate	17.3 grams.
Sodium citrate	173.0 grams.
Sodium carbonate	100.0 grams.

Distilled water to make 1 liter.

With the aid of heat dissolve the sodium citrate and carbonate in about 600 c.c. of water. Pour (through a folded filter paper if necessary) into a glass graduate and make up to 850 c.c. Dissolve the cupric sulphate in about 100 c.c. of water and make up to 150 c.c. Pour the carbonate-citrate solution into a large beaker or casserole and add the cupric sulphate solution slowly, with constant stirring. The mixed solution is ready for use and does not deteriorate upon long standing.

Benedict's solution as used in the *quantitative determination* of sugar consists of *three* separate solutions, the two mentioned under *First Modification* and in addition a *potassium ferro-thiocyanate solution.* This third solution contains 15 grams of potassium ferrocyanide, 62.5 grams of potassium thiocyanate and 50 grams of anhydrous sodium carbonate dissolved in water and made up to 500 c.c. In preparing the Benedict's solution for *quantitative* work the *three* solutions mentioned are combined in equal parts.

Benedict's Sulphur Reagent.

Sodium or potassium chlorate	50 grams.
Distilled water to	1000 c.c.
Crystallized copper nitrate, sulphur-free or of known sulphur content	200 grams.

Black's Reagent.[1]—Made by dissolving 5 grams of ferric chloride and 0.4 gram of ferrous chloride in 100 c.c. of water.

Boas' Reagent.[2]—Dissolve 5 grams of resorcin and 3 grams of sucrose in 100 c.c. of 95 per cent alcohol.

Bonnano's Reagent.—Dissolve 2 grams of sodium nitrite in 100 c.c. of concentrated hydrochloric acid.

Bottu's Reagent.—To 3.5 grams of *o*-nitrophenylpropiolic acid add 5 c.c. of a freshly prepared 10 per cent solution of sodium hydroxide and make the volume of the solution one liter with distilled water.

[1] Black's reaction, page 327.
[2] Test for free acid, page 120.

Congo Red.[1]—Dissolve 0.5 gram of congo red in 90 c.c. of water and add 10 c.c. of 95 per cent alcohol.

Cross and Bevan's Reagent.—Combine *two* parts of concentrated hydrochloric acid and *one* part of zinc chloride *by weight.*

Ehrlich's Diazo Reagent.[2]—Two separate solutions should be prepared and mixed in definite proportions when needed for use.

(*a*) Five grams of sodium nitrite dissolved in 1 liter of distilled water.

(*b*) Five grams of sulphanilic acid and 50 c.c. of hydrochloric acid in 1 liter of distilled water.

Solutions *a* and *b* should be preserved in well-stoppered vessels and mixed in the proportion 1 : 50 when required. Green asserts that greater delicacy is secured by mixing the solutions in the proportion 1 : 100. The sodium nitrite deteriorates upon standing and becomes unfit for use in the course of a few weeks.

Esbach's Reagent.[3]—Dissolve 10 grams of picric acid and 20 grams of citric acid in 1 liter of water.

Fehling's Solution.[4]—Fehling's solution is composed of two definite solutions—a cupric sulphate solution and an alkaline tartrate solution, which may be prepared as follows:

Cupric sulphate solution = 34.65 grams of cupric sulphate dissolved in water and made up to 500 c.c.

Alkaline tartrate solution = 125 grams of potassium hydroxide and 173 grams of Rochelle salt dissolved in water and made up to 500 c.c.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

Ferric Alum Solution.[5]—A cold saturated solution.

Folin-Shaffer Reagent.[6]—This reagent consists of 500 grams of ammonium sulphate, 5 grams of uranium acetate, and 60 c.c. of 10 per cent acetic acid in 650 c.c. of distilled water.

Furfurol Solution.[7]—Add 1 c.c. of furfurol to 1000 c.c. of distilled water.

Gallic Acid Solution.[8]—A saturated alcoholic solution.

[1] Test for free acid, page 121.
[2] Ehrlich's diazo reaction, page 336.
[3] Esbach's method, page 361.
[4] Fehling's method, page 362. Fehling's test, pages 27 and 303.
[5] Volhard-Arnold method, page 390.
[6] Folin-Shaffer method, page 366.
[7] Mylius's modification of Pettenkofer's test, pages 153 and 320. v. Udránsky's test, pages 153 and 321.
[8] Gallic acid test, page 220.

Gies' Biuret Reagent.—This reagent consists of 10 per cent KOH solution to which enough 3 per cent $CuSO_4$ solution has been added to impart a slight though distinct blue color to the clear liquid. The $CuSO_4$ should be added drop by drop with thorough shaking after each addition.

Guaiac Solution.[1]—Dissolve 0.5 gram of guaiac resin in 30 c.c. of 95 per cent alcohol.

Günzberg's Reagent.[2]—Dissolve 2 grams of phloroglucin and 1 gram of vanillin in 100 c.c. of 95 per cent alcohol.

Hammarsten's Reagent.[3]—Mix 1 volume of 25 per cent nitric acid and 19 volumes of 25 per cent hydrochloric acid and add 1 volume of this acid mixture to 4 volumes of 95 per cent alcohol. It is preferable that the acid mixture be prepared in advance and allowed to stand until yellow in color before adding it to the alcohol.

Hopkins-Cole Reagent.[4]—To one liter of a saturated solution of oxalic acid add 60 grams of sodium amalgam and allow the mixture to stand until the evolution of gas ceases. Filter and dilute with 2–3 volumes of water.

Hopkins-Cole Reagent (Benedict's Modification).—Ten grams of powdered magnesium are placed in a large Erlenmeyer flask and shaken up with enough distilled water to liberally cover the magnesium. Two hundred and fifty cubic centimeters of a cold, saturated solution of oxalic acid is now added slowly. The reaction proceeds very rapidly and with the liberation of much heat, so that the flask should be cooled under running water during the addition of the acid. The contents of the flask are shaken after the addition of the last portion of the acid and then poured upon a filter, to remove the insoluble magnesium oxalate. A little wash water is poured through the filter, the filtrate acidified with acetic acid to prevent the partial precipitation of the magnesium on long standing, and made up to a liter with distilled water. This solution contains only the magnesium salt of glyoxylic acid.

Hypobromite Solution.[5]—The ingredients of this solution should be prepared in the form of *two* separate solutions which may be united as needed.

(*a*) Dissolve 125 grams of sodium bromide in water, add 125 grams of bromine and make the total volume of the solution 1 liter.

[1] Guaiac test, pages 174, 191 and 317.
[2] Test for free acid, page 120.
[3] Hammarsten's reaction, pages 152 and 319.
[4] Hopkins-Cole reaction, page 89.
[5] Methods for determination of urea, page 369.

(*b*) A solution of sodium hydroxide having a specific gravity of 1.25. This is approximately a 22.5 per cent solution.

Preserve both solutions in rubber-stoppered bottles and when needed for use mix one volume of solution *a*, one volume of solution *b*, and 3 volumes of water.

Iodine Solution.[1]—Prepare a 2 per cent solution of potassium iodide and add sufficient iodine to color it a deep yellow.

Jolles' Reagent.[2]—This reagent has the following composition:

Succinic acid	40 grams.
Mercuric chloride	20 grams.
Sodium chloride	20 grams.
Distilled water	1000 grams.

Kraut's Reagent.[3]—Dissolve 272 grams of potassium iodide in water and add 80 grams of bismuth subnitrate dissolved in 200 grams of nitric acid (sp. gr. 1.18). Permit the potassium nitrate to crystallize out, then filter it off and make the filtrate up to 1 liter with water.

Lugol's Solution.[4]—Dissolve 4 grams of iodine and 6 grams of potassium iodide in 100 c.c. of distilled water.

Magnesia Mixture.[5]—Dissolve 175 grams of magnesium sulphate and 350 grams of ammonium chloride in 1400 c.c. of distilled water. Add 700 grams of concentrated ammonium hydroxide, mix thoroughly, and preserve the mixture in a glass-stoppered bottle.

Millon's Reagent.[6]—Digest 1 part (by weight) of mercury with 2 parts (by weight) of nitric acid (sp. gr. 1.42) and dilute the resulting solution with 2 volumes of water.

Molisch's Reagent.[7]—A 15 per cent alcoholic solution of α-naphthol.

Molybdic Solution.[8]—Molybdic solution is prepared as follows, the parts being *by weight:*

Molybdic acid	1 part.
Ammonium hydroxide (sp. gr. 0.96)	4 parts.
Nitric acid (sp. gr. 1.2)	15 parts.

Moreigne's Reagent.[9]—Combine 20 grams of sodium tungstate, 10 grams of phosphoric acid (sp. gr. 1.13) and 100 c.c. of water.

[1] Iodine test, page 45.
[2] Jolles' reaction, pages 96 and 310.
[3] Rosenheim's bismuth test for choline, page 248.
[4] Gunning's iodoform test, page 327, and Bardach's reaction, page 92.
[5] Sodium hydroxide and potassium nitrate fusion method for determination of total phosphorus, page 384.
[6] Millon's reaction, page 88.
[7] Molisch's reaction, page 22.
[8] Sodium hydroxide and potassium nitrate fusion method for determination of total phosphorus, page 384.
[9] Moreigne's reaction, page 269.

Boil the mixture for twenty minutes, add water to make the volume of the solution equivalent to the original volume, and acidify with hydrochloric acid.

Morner's Reagent.[1]—Thoroughly mix 1 volume of formalin, 45 volumes of distilled water, and 55 volumes of concentrated sulphuric acid.

Nakayama's Reagent.[2]—Prepared by combining 99 c.c. of alcohol and 1 c.c. of fuming hydrochloric acid containing 4 grams of ferric chloride per liter.

Neutral Olive Oil.[3]—Shake ordinary olive oil with a 10 per cent solution of sodium carbonate, extract the mixture with ether, and remove the ether by evaporation. The residue is *neutral* olive oil.

Nylander's Reagent.[4]—Digest 2 grams of bismuth subnitrate and 4 grams of Rochelle salt in 100 c.c. of a 10 per cent solution of potassium hydroxide. The reagent should then be cooled and filtered.

Obermayer's Reagent.[5]—Add 2–4 grams of ferric chloride to a liter of hydrochloric acid (sp. gr. 1.19).

Oxalated Plasma.[6]—Allow arterial blood to run into an equal volume of 0.2 per cent ammonium oxalate solution.

Para-dimethylaminobenzaldehyde Solution.[7]—This solution is made by dissolving 5 grams of para-dimethylaminobenzaldehyde in 100 c.c. of 10 per cent sulphuric acid.

Para-phenelenediamine Hydrochloride Solution.[8]—Two grams dissolved in 100 c.c. of water.

Phenolphthalein.[9]—Dissolve 1 gram of phenolphthalein in 100 c.c. of 95 per cent alcohol.

Phenylhydrazine Mixture.[10]—This mixture is prepared by combining 1 part of phenylhydrazine-hydrochloride and 2 parts of sodium acetate *by weight.* These are thoroughly mixed in a mortar.

Phenylhydrazine-acetate Solution.[11]—This solution is prepared by mixing 1 volume of glacial acetic acid, 1 volume of water, and 2 volumes of phenylhydrazine (the base).

[1] Mörner's test, page 82.
[2] Nakayama's reaction, pages 151 and 319.
[3] Emulsification of fats, page 133.
[4] Nylander's test, pages 29 and 306.
[5] Obermayer's test, page 275.
[6] Experiments on blood plasma, page 106.
[7] Herter's para-dimethylaminobenzaldehyde reaction, page 166.
[8] Detection of hydrogen peroxide, page 221.
[9] Töpfer's method, page 408.
[10] Phenylhydrazine reaction, pages 23 and 300.
[11] Phenylhydrazine reaction, pages 23 and 300.

Purdy's Solution.[1]—Purdy's solution has the following composition:

Cupric sulphate	4.752	grams.
Potassium hydroxide	23.5	grams.
Ammonia (U. S. P., sp. gr. 0.9)	350.0	c.c.
Glycerol	38.0	c.c.
Distilled water, to make total volume 1 liter.		

Roberts' Reagent.[2]—Mix 1 volume of concentrated nitric acid and 5 volumes of a saturated solution of magnesium sulphate.

Rosenheim's Iodo-Potassium Iodide Solution.[3]—Dissolve 2 grams of iodine and 6 grams of potassium iodide in 100 c.c. of water.

Salted Plasma.[4]—Allow arterial blood to run into an equal volume of a saturated solution of sodium sulphate or a 10 per cent solution of sodium chloride. Keep the mixture in the cold room for about 24 hours.

Schiff's Reagent.[5]—This reagent consists of a mixture of three volumes of concentrated sulphuric acid and one volume of 10 per cent ferric chloride.

Schweitzer's Reagent.[6]—Add potassium hydroxide to a solution of cupric sulphate which contains some ammonium chloride. Filter off the precipitate of cupric hydroxide, wash it, and bring 3 grams of the moist cupric hydroxide into solution in a liter of 20 per cent ammonium hydroxide.

Seliwanoff's Reagent.[7]—Dissolve 0.05 gram of resorcin in 100 c.c. of dilute (1:2) hydrochloric acid

Sherrington's Solution.[8]—This solution possesses the following formula:

Methylene-blue	0.1	gram.
Sodium chloride	1.2	grams.
Neutral potassium oxalate	1.2	grams.
Distilled water	300.0	grams.

Sodium Acetate Solution.[9]—Dissolve 100 grams of sodium acetate in 800 c.c. of distilled water, add 100 c.c. of 30 per cent acetic acid to the solution, and make the volume of the mixture up to 1 liter with distilled water.

Sodium Alizarin Sulphonate.[10]—Dissolve 1 gram of sodium alizarin sulphonate in 100 c.c. of water.

[1] Purdy's method, page 364.
[2] Roberts' ring test, pages 95 and 310.
[3] Rosenheim's periodide test, page 248.
[4] Experiments on blood plasma, page 196.
[5] Schiff's reaction, pages 155 and 247.
[6] Schweitzer's solubility test, page 40.
[7] Seliwanoff's reaction, pages 34 and 333.
[8] "Blood counting," page 208.
[9] Uranium acetate method, page 383.
[10] Töpfer's method, page 408.

Sodium Sulphide Solution.[1]—Saturate a 1 per cent solution of sodium hydroxide with hydrogen sulphide gas and add an equal volume of 1 per cent sodium hydroxide.

Solera's Test Paper.[2]—Saturate a good quality of filter paper with 0.5 per cent starch paste to which has been added sufficient iodic acid to make a 1 per cent solution of iodic acid and allow the paper to dry in the air. Cut it in strips of suitable size and preserve for use.

Spiegler's Reagent.[3]—This reagent has the following composition:

Tartaric acid	20 grams.
Mercuric chloride	40 grams.
Glycerol	100 grams.
Distilled water	1000 grams.

Standard Ammonium Thiocyanate Solution.[4]—This solution is made of such a strength that 1 c.c. of it is equal to 1 c.c. of the standard argentic nitrate solution mentioned below. To prepare the solution dissolve 12.9 grams of ammonium thiocyanate, NH_4SCN, in a little less than a liter of water. In a small flask place 20 c.c. of the standard argentic nitrate solution, 5 c.c. of a cold saturated solution of ferric alum and 4 c.c. of nitric acid (sp. gr. 1.2), add water to make the total volume 100 c.c., and thoroughly mix the contents of the flask. Now run in the ammonium thiocyanate solution from a burette until a permanent *brown* tinge is produced. This is the end-reaction and indicates that the last trace of argentic nitrate has been precipitated. Take the burette reading and calculate the amount of water necessary to use in diluting the ammonium thiocyanate in order that 10 c.c. of this solution may be exactly equal to 10 c.c. of the argentic nitrate solution. Make the dilution and titrate again to be certain that the solution is of the proper strength.

Standard Argentic Nitrate Solution.[5]—Dissolve 29.06 grams of argentic nitrate in 1 liter of distilled water. Each cubic centimeter of this solution is equivalent to 0.01 gram of sodium chloride or to 0.006 gram of chlorine.

Standard Uranium Acetate Solution.[6]—Dissolve 35.461 grams of uranium acetate in 1 liter of water. One c.c. of such a solution should be equivalent to 0.005 gram of P_2O_5, phosphoric anhydride.

[1] Krüger and Schmidt's method, pages 368 and 399.
[2] Solera's reaction, page 56.
[3] Spiegler's ring test, pages 96 and 310.
[4] Volhard-Arnold method, page 390, and Clark's modification of Dehn's method, page 388.
[5] Volhard-Arnold method, page 390, Mohr's method, page 389, and Clark's modification of Dehn's method, page 388.
[6] Uranium acetate method, page 383.

This solution may be standardized as follows: To 50 c.c. of a standard solution of disodium hydrogen phosphate, of such a strength that the 50 c.c. contains 0.1 gram of P_2O_5, add 5 c.c. of the sodium acetate solution mentioned on p. 417 and titrate with the uranium solution to the correct end-reaction as indicated in the method proper on p. 383. Inasmuch as 1 c.c. of the uranium solution should precipitate 0.005 gram of P_2O_5, exactly 20 c.c. of the uranium solution should be required to precipitate the 50 c.c. of the standard phosphate solution. It the two solutions do not bear this relation to each other they must be brought into the proper relation by diluting the uranium solution with distilled water or by increasing its strength.

Starch Iodide Solution.[1]—Mix 0.1 gram of starch powder with *cold* water in a mortar and pour the suspended starch granules into 75–100 c.c. of boiling water, stirring continuously. Cool the starch paste, add 20–25 grams of potassium iodide and dilute the mixture to 50 c.c. This solution deteriorates upon standing, and therefore must be freshly prepared as needed.

Starch Paste.[2]—Grind 2 grams of starch powder in a mortar with a small amount of water. Bring 200 c.c. of water to the boiling-point and add the starch mixture from the mortar with continuous stirring. Bring again to the boiling-point and allow it to cool. This makes an approximate 1 per cent starch paste which is a very satisfactory strength for general use.

Stokes' Reagent.[3]—A solution containing 2 per cent ferrous sulphate and 3 per cent tartaric acid. When needed for use a small amount should be placed in a test-tube and ammonium hydroxide added until the precipitate which forms on the first addition of the hydroxide has entirely dissolved. This produces *ammonium ferrotartrate* which is a reducing agent.

Suspension of Manganese Dioxide.[4]—Made by heating a 0.5 per cent solution of potassium permanganate with a little alcohol until it is decolorized.

Tanret's Reagent.[5]—Dissolve 1.35 grams of mercuric chloride in 25 c.c. of water, add to this solution 3.32 grams of potassium iodide dissolved in 25 c.c. of water, then make the total solution up to 60 c.c. with distilled water and add 20 c.c. of glacial acetic acid to the mixture.

[1] Fehling's method, page 362.
[2] Fehling's method, page 362
[3] Hæmoglobin, page 199. Hæmochromogen, page 202.
[4] Krüger and Schmidt's method, pages 368 and 399.
[5] Tanret's test, pages 96 and 311.

Tincture of Iodine.[1]—Dissolve 70 grams of iodine and 50 grams of potassium iodide in 1 liter of 95 per cent alcohol.

Toison's Solution.[2]—This solution has the following formula:

Methyl violet	0.025	gram.
Sodium chloride	1.0	gram.
Sodium sulphate	8.0	grams.
Glycerol	30.0	grams.
Distilled water	160.0	grams.

Töpfer's Reagent.[3]—Dissolve 0.5 gram of di-methylamino-azobenzene in 100 c.c. of 95 per cent alcohol.

Tropæolin OO.[4]—Dissolve 0.05 gram of tropæolin OO in 100 c.c. of 50 per cent alcohol.

Uffelmann's Reagent.[5]—Add a 5 per cent solution of ferric chloride to a 1 per cent solution of carbolic acid until an amethyst-blue color is obtained.

[1] Smith's test, pages 152 and 319.
[2] "Blood counting," page 208.
[3] Töpfer's method, page 408.
[4] Test for free acid, page 120.
[5] Uffelmann's reaction, page 125.

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